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TITLE: ROLE OF NATURAL KILLER T CELLS IN IMMUNOGENIC CHEMOTHERAPY FOR BREAST
CANCER

PRINCIPAL INVESTIGATOR: Dr Christophe Paget

CONTRACTING ORGANIZATION: University of Melbourne
Parkville, VIC, 3052 Australia

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14. ABSTRACT During this second year of investigation, we have confirmed the important role of $\gamma\delta$ T cells in the efficacy of immunogenic chemotherapy using anthracyclines. In the meantime, the NKT cells seem to be dispensable for this effect at least in this particular situation. Moreover, we have also identified a population of $\gamma\delta$ T cells with a unique phenotype never described before (CD3 ^{bright}). Interestingly, this population has a clear intrinsic ability to produce IL-17 compared to any other $\gamma\delta$ T cells and are represented at a high frequency in breast cancer tumor environment. We have highlighted several key homeostatic factors on this population and identified their precise phenotype. Of interest a human $\gamma\delta$ T cell population display a similar phenotype (CD3 ^{bright}) with $\gamma\delta$ T17-like cell hallmarks. Our future efforts will focus on this population and their putative role in immunogenic chemotherapy for breast cancer.					
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Introduction:

The role of immunity in controlling and regulating development of breast cancer is largely accepted. Recent studies have also demonstrated that some common chemotherapy used for breast cancer treatment relies on a competent immune system of the host to achieve an optimal response¹⁻³. Interestingly seminal findings emphasized on the importance of various components of the immune system in this phenomenon^{1,4-6}. Recently, we and others have demonstrated that IL-17-producing $\gamma\delta$ T cells are well positioned and equipped to participate in this effect.

Body:

As assigned in the previous report (Year 1), we have identified an unpreviously described subset of $\gamma\delta$ T cells displaying a high level of CD3 in comparison to the remaining population and pre-programmed to secrete high amounts of IL-17. In addition to the phenotype presented in the last report, we have formerly highlighted several key factors involved in the homeostasis of the CD3^{bright} $\gamma\delta$ T cell population. In this context, CD3^{bright} $\gamma\delta$ T cells are largely influenced by the microbiota and are fully dependent on the expression of ROR γ t, a mandatory transcription factor for IL-17 production (**Figure 1**).

Using an approach of single cell PCR, we have demonstrated that CD3^{bright} $\gamma\delta$ T cells express a fully germline encoded invariant V γ 6V δ 1 TCR (**Figure 2**).

Of upmost interest, similar to mice, we observed a subset of $\gamma\delta$ T cells displaying a bright expression of CD3 in the bone marrow, but not in the peripheral blood lymphocytes of healthy donors (**Figure 3A**). Of interest, these cells displayed hallmarks of human IL-17-producing cells, uniformly expressing CD161⁷ (**Figure 3B**). Similar to the observations made in mice, human CD3^{bright} $\gamma\delta$ T cells expressed high levels of IL-7R and IL-18R compared to the conventional population and harboured a phenotype of memory cells (**Figure 3B**). Finally, culture of purified $\gamma\delta$ T cell subsets with a pre-coated anti-CD3 induced $\gamma\delta$ T cell expansion. Interestingly, association

with a recombinant IL-7, a selective growth factor for $\gamma\delta$ T17 cells⁸ favoured CD3^{bright} $\gamma\delta$ T cell expansion but had a minimal effect on conventional $\gamma\delta$ T cells (**Figure 3C**). This observation is of high importance considering that identification of human IL-17-producing $\gamma\delta$ T cells in healthy donors remains challenging⁹, despite the fact that these cells have been associated with various immune responses¹⁰.

A manuscript has been submitted to *PNAS* and is currently under review.

Having this paper submitted and as mentioned in the previous report, we will focus our efforts in investigating the contribution of this population in development of breast cancer or immunogenic chemotherapy for breast cancer considering their specialized function (IL-17 production) and their abundance in breast cancer tumors. Of note, this population is also found at a high frequency in various solid tumors (transplantable and spontaneous) in mice from different background (C57BL/6 and BALB/C) (**Figure 5**).

Moreover, all points from the original proposal, and as discussed in the previous report, will be tested in the coming next months. In February, I have quitted my position at Melbourne University and joined the INSERM in France. Recently I have been offered a tenured position and the transfer of the award is still pending between Melbourne University and INSERM.

Key Research Accomplishment:

- CD3^{bright} $\gamma\delta$ T cells express a **unique V γ 6V δ 1 TCR**.
- Discovery of a **similar population of CD3^{bright} $\gamma\delta$ T cells in humans also associated with a signature of IL-17-producing cells**.
- **Submission of an original manuscript to *PNAS* describing a new way to define mouse and human $\gamma\delta$ T cells with high capacity to produce IL-17.**

Conclusions:

We have identified a population of $\gamma\delta$ T cells both in mouse and humans with a unique phenotype never described before (CD3^{bright}). Interestingly, this population has a potent intrinsic ability to produce IL-17 compared to the other $\gamma\delta$ T cells and is enriched in breast cancer tumor environment. Our future efforts will focus on this population and their putative role in immunogenic chemotherapy for breast cancer and more generally in the development of breast cancer.

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Appendices:

Figures shown in this section are unpublished.

Figure 1

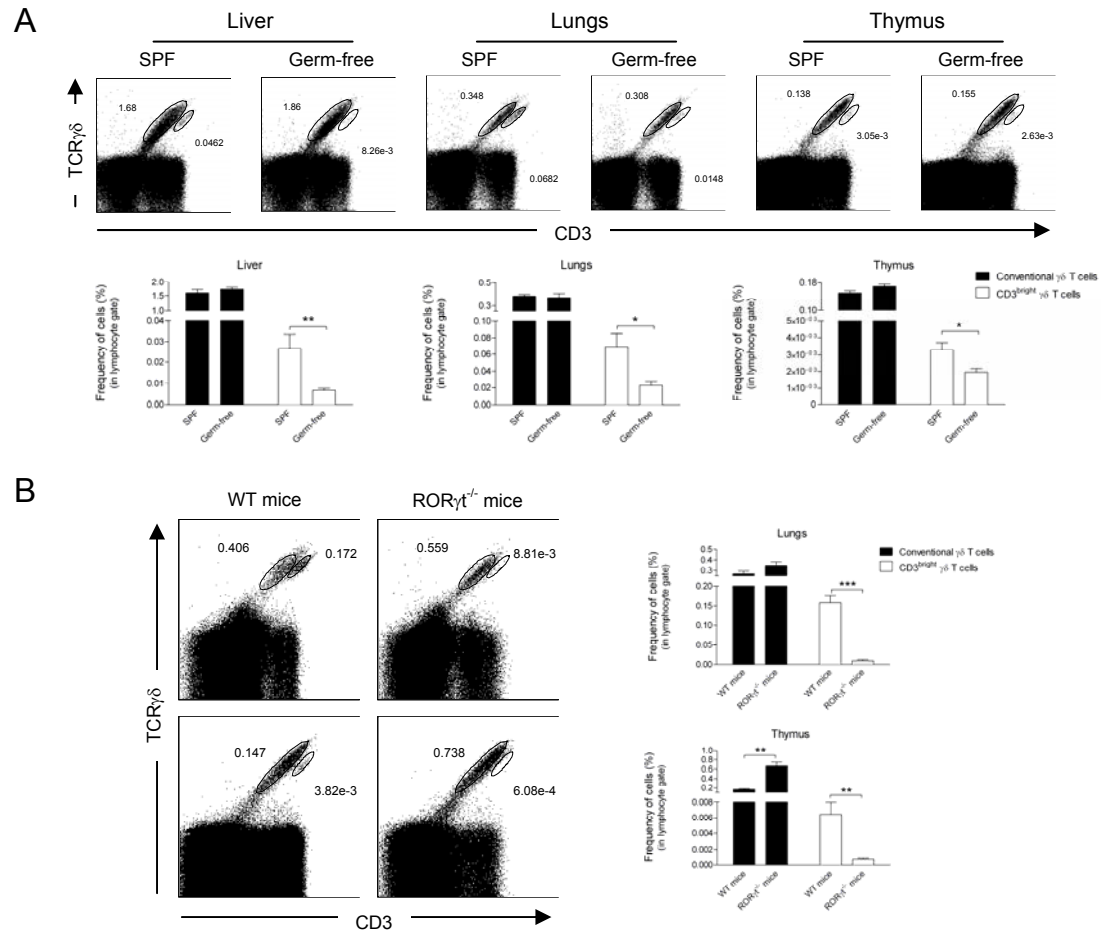
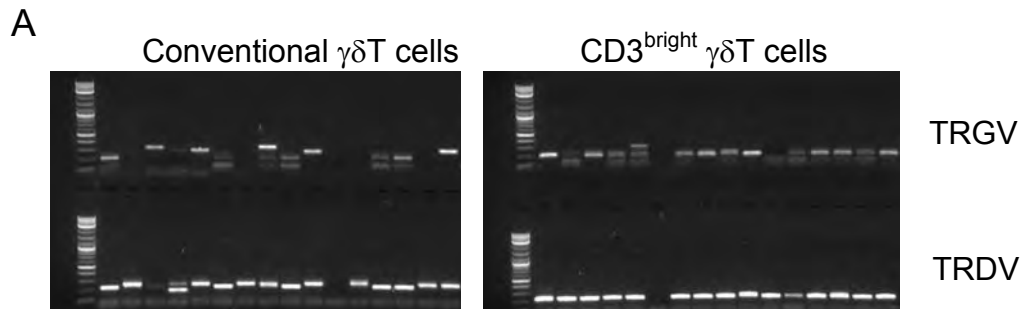


Figure 1: Factors involved in CD3^{bright} $\gamma\delta$ T cell homeostasis. *A*, frequency of $\gamma\delta$ T cell subsets based on CD3 expression was analyzed by flow cytometry in mice bred either in GF or SPF conditions. The percentage of $\gamma\delta$ T cell subsets is shown (*upper panel*). The average \pm SEM of $\gamma\delta$ T cell subset frequencies pooled from two independent experiments is shown in the *lower panel*. Of note, the total number of cells in each organ was not different between GF and SPF mice. Differences in mean were analyzed using a Mann-Whitney test. *, $p < 0.05$ and **, $p < 0.01$. *B*, frequency of $\gamma\delta$ T cell subsets was analyzed by flow cytometry in WT vs ROR γ t^{-/-} mice. The percentage of $\gamma\delta$ T cell subsets is shown (*left panel*). The mean \pm SEM of $\gamma\delta$ T cell subset frequencies pooled from two independent experiments is shown in the *right panel*. Differences in mean were analyzed using a Mann-Whitney test. **, $p < 0.01$ and ***, $p < 0.001$.

Figure 2



B

Subsets	#	V γ	CDR3 γ	J γ	V δ	CDR3 δ	D δ	J δ
Conventional $\gamma\delta$ T cells	1	2	CAVWMRYSSGFHKVF	2	5	CASGYSWHIGGIRSSSTDKLVF	2	1
	2		-	-	5	CASGVYGTLYRRDTKLVF	1	1
	3		-	-	4	CALMERGAFFPYRRDKGTDKLVF	1	1
	4		-	-	5	CASGYIGGIRATDKLVF	2	1
	5	2	CAVCRLARKF#KVF	2	2	CALMLYGLLSEGPTTDKLVF	1	1
CD3 ^{bright} $\gamma\delta$ T cells	1	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2
	2	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2
	3	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2
	4	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2
	5	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2
	6	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2
	7	6	CACWDSSGCHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2
	8	6	CACWDSSGFHKVF	1	1	CGSDIGGSSWDTRQMFF	2	2

Figure 2: CD3^{bright} $\gamma\delta$ T cells have an invariant V γ 6V δ 1 TCR. **A**, PCR analysis of cDNA isolated from single cell-sorted CD3^{bright} and conventional $\gamma\delta$ T cells and amplified with a set of primers specific for each TCR γ V-gene segment or the corresponding constant region (C γ) (upper row) or specific for each TCR δ V-gene segment or the corresponding constant region (C δ) (lower row). **B**, Sequences for CD3^{bright} and conventional $\gamma\delta$ T TCRs including diversity (D) (for δ chains), junction (J) and CDR3 region.

Figure 3

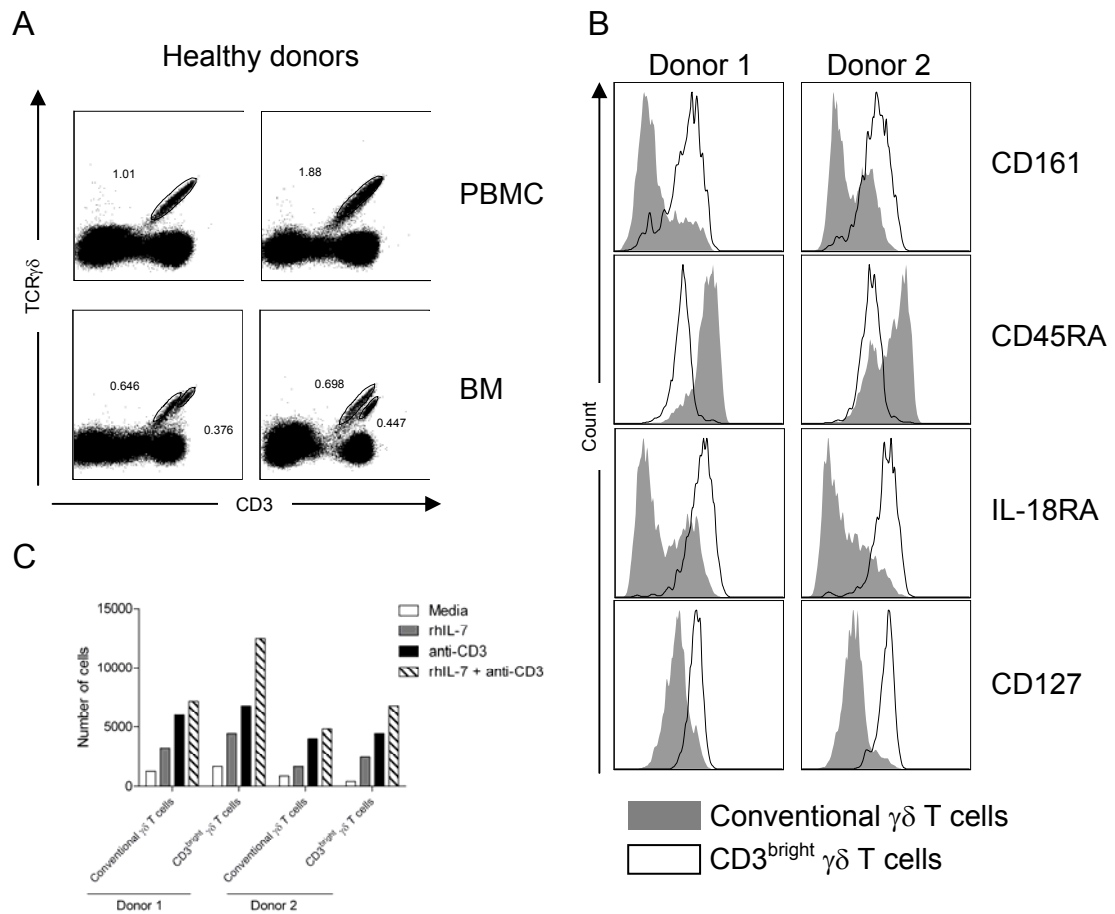


Figure 3: Human CD3^{bright} but not conventional $\gamma\delta$ T cells display characteristics of IL-17-producing cells. A, Single cell suspensions of peripheral blood mononuclear cells (PBMC) or bone marrow (BM) from healthy donors were analyzed by flow cytometry for CD3 and TCR δ expression. B, $\gamma\delta$ T subsets (based on CD3 expression) were stained with different combinations of antibodies and were analyzed for CD161, CD45RA, IL-18RA and CD127 differential expression. C, purified $\gamma\delta$ T subsets (based on CD3 expression) from bone marrow of two donors were cultured for 4 days in presence of recombinant IL-7 (20 ng/ml) or/and pre-coated anti-CD3 (0.5 μ g/ml/clone: OKT3). At end point, number of cells was counted under the microscope.

CD3 expression reveals a gamma/delta T cell subset with specialized functions

Christophe Paget^{a,b,c,1}, Melvyn T. Chow^{a,b,d}, Nicholas A. Gherardin^{a,b,g}, Paul A. Beavis^{a,b}, Adam P. Uldrich^g, Helene Duret^{a,b}, Maya Hassane^c, Fernando Souza-Fonseca-Guimaraes^d, Geoffrey R. Hill^{d,e}, Paul Neeson^{a,b}, David S. Ritchie^{a,b}, François Trottein^c, Gabrielle T. Belz^h, Dale I. Godfrey^g and Mark J. Smyth^{a,b,d,f,1}

^aCancer Immunology Program, Peter MacCallum Cancer Centre, St. Andrews Place, East Melbourne, Victoria 3002, Australia.

^bSir Peter MacCallum Department of Oncology and Department of Pathology, University of Melbourne, Parkville 3010, Australia.

^cINSERM U1019, Centre d'Infection et d'Immunité de Lille, Institut Pasteur de Lille, 59000 Lille, France.

^dQIMR Berghofer Medical Research Institute, Herston, Queensland 4006, Australia.

^eDepartment of Bone Marrow Transplantation, Royal Brisbane Hospital, Herston, Australia.

^fSchool of Medicine, University of Queensland, Herston, Queensland 4006, Australia.

^gDepartment of Microbiology and Immunology, University of Melbourne, Parkville, VIC 3010, Australia.

^hDivision of Molecular Immunology, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, 3052, Australia.

¹Correspondence: christophe.paget@pasteur-lille.fr or mark.smyth@qimrberghofer.edu.au

Running title: Level of CD3 expression as a marker of $\gamma\delta$ T17

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Key-words: $\gamma\delta$ T cells, IL-17, IL-23, IL-1 β , invariant TCR, microbiome, ROR γ t, alpha-galactosylceramide, lipopolysaccharide, innate immunity, neutrophils, mucosal immunity.

Conflict of interest: All authors declare that they have no conflict of interest

Abbreviations: α -GalCer, α -galactosylceramide; CBA, cytometric bead array; NLRP3, NOD-like receptor family, pyrin domain containing 3; TCR, T cell receptor; WT, wildtype.

Summary

Interleukin-17A is a pro-inflammatory cytokine that plays an important role in a wide range of immune responses including infection, allergy and auto-immunity. $\gamma\delta$ T cells are recognized as secretors of IL-17, but now we define the remarkable ability of a CD3^{bright} V γ 6V δ 1 $\gamma\delta$ T cell subset with effector memory phenotype to rapidly produce IL-17A, but not IFN- γ . These cells are widely distributed with a preferential representation in the lungs and do not develop properly in absence of ROR γ t expression or in germ-free mice. Interestingly this population responded promptly to various stresses in a mechanism involving IL-23 and NLRP3-inflammasome-dependent IL-1 β . Finally, we demonstrated that pulmonary CD3^{bright} $\gamma\delta$ T cells expanded/were recruited and produced an early substantial amount of IL-17 in response to pneumococcal infection. Given the versatile role of IL-17 in pulmonary immunity, the prevalence of this population in lungs suggests a pivotal role of this $\gamma\delta$ T cell subset in respiratory disorders.

Introduction

$\gamma\delta$ T cells have diverse functional specializations and promptly produce copious amounts of immunoregulatory cytokines, conferring on them a pivotal role in a broad range of pathologies including infection, allergy, auto-immunity and cancer (1). In particular, mouse $\gamma\delta$ T cells are known as major early IL-17 producers (2). In this context, IL-17-producing $\gamma\delta$ T cells ($\gamma\delta$ T17) may be either considered beneficial or harmful to the host depending upon to the disease. For example, IL-17 is an important anti-microbial cytokine predominantly due to its ability to induce chemokine release, growth factors, and adhesion molecules; and to indirectly increase neutrophilia through epithelial and stromal cell-dependent soluble factors (3, 4). This feature is of particular interest during mucosal infections in which IL-17 has been demonstrated to be critical in the control of tuberculosis, candidiasis, *Escherichia coli* and *Staphylococcus aureus* infections (5, 6). In these models, $\gamma\delta$ T cells have been reported to be a major source of this cytokine (5, 7-9). On the other hand, IL-17 has been proposed to participate in the development of a diversity of inflammation or auto-immune disorders including arthritis, asthma and EAE (6) and similarly, $\gamma\delta$ T17 cells play an important part in these processes (10). Finally, the role of IL-17 in tumorigenesis or control of tumor progression has recently been studied but remains controversial (11). However, $\gamma\delta$ T17 cells appear to be important in eliciting a positive outcome during immunogenic anti-cancer chemotherapy (12). In humans, little is known about the real functional relevance of $\gamma\delta$ T17 cells in IL-17-dependent pathologies (13) despite the fact that IL-17-production by $\gamma\delta$ T cells has been detected during infections and chronic inflammation (14-17). However, characterization, isolation and activation of these cells in humans remains extremely challenging (14).

Numerous recent studies have also focused on the factors influencing $\gamma\delta$ T17 cell differentiation, homeostasis and activation (10, 18). $\gamma\delta$ T17 cells express a fairly restricted TCR repertoire ($V\gamma 4^+$ and $V\gamma 6^+$ chain predominantly) (9, 19-21) and originate from fetal thymus without signs of TCR-dependent positive selection (19, 22). Within the thymus, $\gamma\delta$ T17 cells have a pre-programmed IL-17-producing phenotype, although these cells do not require the transcription factors ROR γ t and STAT3 to develop (23), even though STAT3 remains mandatory for their activation in periphery. Moreover, the strength of engagement with cognate antigen(s) (Ag) by $\gamma\delta$ TCRs in the thymus seems to be the key factor influencing the commitment towards IFN- γ - or IL-17-producing $\gamma\delta$ T cell lineages (19, 22, 24). In this context, absence (or weak) TCR ligation seems to determine a transcriptional program leading to the development of $\gamma\delta$ T17 cells (19, 22, 24). In addition, Notch/Hes1 signaling pathways are involved in the development and homeostasis of $\gamma\delta$ T17 cells (23). While IL-6 and IL-23 are dispensable for the development of $\gamma\delta$ T17 cells (23, 25), TGF- β may be required since *Tgfb*^{-/-} mice show a significant reduction in thymic $\gamma\delta$ T17 cells (26). Whilst IL-23 is not required for $\gamma\delta$ T17 cell development, this cytokine along with IL-1 β , IL-6, IL-7 and IL-18, potently promotes $\gamma\delta$ T17 cell functions (expansion and/or cytokine production) (25, 27-29).

Here, we demonstrate in mice that the level of cell surface CD3 expression reveals a population of $\gamma\delta$ T cells bearing a clonal $V\gamma 6V\delta 1$ TCR associated with a great ability to secrete IL-17. We characterize some key homeostatic factors and activation mechanisms for this population *in vitro* and *in vivo*. This population participates in the early phase of the immune response against respiratory bacterial infections by increasing in absolute number and producing high levels of IL-

17 compared to other acknowledged innate IL-17-producing cells. Given the versatile role of IL-17 in pulmonary immunity, the prevalence of this population in lungs of adult mice suggests a pivotal role of this $\gamma\delta$ T cell subset in respiratory disorders.

Experimental procedures

Mice

Male C57BL/6J wild-type mice were purchased from the Walter and Eliza Hall Institute for Medical Research (WEHI). C57BL/6 TCR δ -deficient (TCR $\delta^{-/-}$) mice, C57BL/6 IL-23p19-deficient (IL-23p19 $^{-/-}$) mice, C57BL/6 IL-12p35-deficient (IL-12p35 $^{-/-}$) mice, C57BL/6 IL-12p40-deficient (IL-12p40 $^{-/-}$) mice, C57BL/6 IL-21-deficient (IL-21 $^{-/-}$) mice, C57BL/6 IL-6-deficient (IL-6 $^{-/-}$) mice, C57BL/6 IL-1R1-deficient (IL-1R1 $^{-/-}$), C57BL/6 NLRP3-deficient (NLRP3 $^{-/-}$) mice, C57BL/6 caspase-1-deficient (ICE $^{-/-}$) mice, and C57BL/6 IL-23R $^{+/+}$ -GFP knock-in (IL-23R-GFP reporter) mice (kindly provided by Vijay Kuchroo, Harvard Medical School, Boston, USA) were bred in house at the Peter MacCallum Cancer Centre (Peter Mac). C57BL/6 ROR γ t $^{+/+}$ -GFP knock-in mice, ROR γ t $^{-/-}$ -GFP knock in (ROR γ t-GFP reporter) mice, Germ-free mice and SPF controls were obtained from WEHI. C57BL/6 MyD88/TRIF-deficient (MyD88 $^{-/-}$ TRIF $^{-/-}$) mice (kindly provided by Dr. Geoff Hill, QIMR Berghofer) and C57BL/6 Fc ϵ RI γ -deficient (Fc ϵ RI $\gamma^{-/-}$) mice (kindly provided by Dr. Mark Hogarth) were bred in house at the QIMR Berghofer Medical Research Institute (QIMR Berghofer). All mice were backcrossed to C57BL/6J at least ten times. Sex-matched mice were used at the ages of 8–10 weeks. All experiments were performed in accordance with the animal ethics guidelines ascribed by the National Health and Medical Research Council of Australia. All experiments were approved by the Peter Mac and QIMR Berghofer Animal Ethics Committees.

Mouse reagents and Abs

α -galactosylceramide (α -GalCer) was from Axxora Life Sciences (San Diego, CA, USA). Lipopolysaccharide (LPS) from E. coli O111:B4 was from Sigma-Aldrich (St. Louis, MO USA).

Monoclonal Abs against mouse CD3 (17A2: PB-conjugated), NK1.1 (PK136: PECy7- or PE-conjugated), anti-C δ (GL3: APC- or FITC-conjugated), CD69 (FN50: FITC-conjugated), IFN- γ (XMG1.2: PE-conjugated), IL-17A (TC11-18H10: PE-conjugated), CD27 (LG.7F9: PECy7-conjugated), TCR β (H57-597: PE-conjugated), CD44 (IM7: FITC-conjugated), CD62L (MEL-14: PE-conjugated), CD127 (A7R34: FITC-conjugated), CD122 (TM- β 1: FITC-conjugated), NKG2D (C7: APC-conjugated), IL-18R α (112614: APC-conjugated), CD121a (35F5: PE-conjugated) and isotype controls were purchased from BD Biosciences (San Diego, CA, USA), BioLegend (San Diego, CA, USA), R&D system (Minneapolis, MN USA), Immunostep (Salamanca, Spain) or eBiosciences (San Diego, CA, USA). Recombinant mouse IL-23, IL-1 β , IL-12p70 and IL-18 were from R&D Systems. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma-Aldrich.

Infection with S. pneumoniae

S. pneumoniae serotype 1 clinical isolate E1586 sequence type ST304 has been described elsewhere (30). Mice were anesthetized and administered i.n. with 2×10^6 live bacteria.

Preparation of splenic, pulmonary and liver cells

Splenic, pulmonary and hepatic mononuclear cells from control, LPS- or α -GalCer-treated mice were prepared as previously described (31). Briefly, lungs and livers were perfused with PBS, excised and finely minced, followed by enzymatic digestion for 30 minutes at 37°C in PBS containing 1 mg/ml collagenase type IV and 1 μ g/ml DNase type I (Roche). After washing, homogenates were resuspended in a 35% Percoll™ gradient, carefully layered onto 70% Percoll™ and centrifuged at 2300 (liver) or 2600 (lungs) rpm without brake at 22°C for 30 min.

The layer at the interface between the two Percoll™ concentrations was carefully aspirated and washed in PBS 2% FCS. Red blood cells were removed with Ammonium-Chloride-Potassium (ACK) lysis buffer.

Flow cytometry and cytospin

Mice were injected with PBS, α -GalCer (2 μ g/mouse i.p. or 500 ng/mouse intranasal (i.n.) route) or *Escherichia coli* LPS (0.5 mg/mouse). Livers, lungs and spleens were harvested at different time points and mononuclear cells (MNCs) were prepared as described above. Golgi Plug/Golgi Stop (BD Biosciences) was added for 2 hrs. Then cell suspensions were blocked in the presence of 2.4G2 prior to staining with appropriate dilutions of APC-conjugated TCR $\gamma\delta$, PB-labeled anti-CD3 and PeCy7-conjugated NK1.1 for 30 min in PBS containing 2% FCS and 0.01% NaN₃. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm™. Fixation/Permeabilization Kit and incubated with PE-conjugated mAb against IFN- γ , IL-17A or matching isotype control mAbs in permeabilization buffer. Cells were acquired and analyzed on either Canto2 or LSR-II cytometer (BD Biosciences CA). FACS analysis was performed with FlowJo (Treestar, OR USA). A morphology-based differential cell count was conducted on cytospin preparations from the bronchoalveolar lavage (BAL) fluid samples and stained with Giemsa solution.

Detection of cytokines

Cytokines were detected using the BD Cytometric Bead Array (CBA) system (BD Bioscience CA). Acquisition was performed on an LSR-II (BD Bioscience). A total of 300 bead events for each cytokine were collected. Analysis was performed using FCAP array software (BD).

Isolation of $\gamma\delta$ T subsets and in vitro assays

Livers, lungs and spleens were harvested from naive mice and cell suspensions were prepared as previously described (31). Red blood cells were lysed with ACK lysis buffer prior to $\gamma\delta$ T cell enrichment with autoMACS (depletion of TCR β^+ , CD19 $^+$ and F4/80 $^+$ cells). Then, $\gamma\delta$ T-enriched cells were sorted using an ARIA2 and purity was always greater than 97%. Of note, the pre-enrichment step does not modulate the ratio between conventional $\gamma\delta$ T cells and CD3^{bright} $\gamma\delta$ T cells. For the stimulation assay, purified cells were cultured for the indicated amount of time in complete media containing a combination of recombinant mouse IL-12p70 (50 pg/ml), IL-23 (1 ng/ml), IL-1 β (1 ng/ml), IL-18 (1 ng/ml), PMA (10 ng/ml) and ionomycin (1 μ g/ml). In some case purified cells have been stimulated with various concentrations of anti-CD3 ϵ (clone 145-2C11) (BD Pharmingen) pre-coated on the culture plates.

Single-cell PCR

For single-cell PCR, cDNA from sorted mouse and human $\gamma\delta$ T cell subsets was generated as previously described (11) and then was amplified by two rounds of semi-nested PCR with sense primers for C γ , C δ , V γ and V δ regions each used with the C γ and/or C δ antisense primers (Table 1). PCR products were separated by electrophoresis through a 1.5% agarose gel and were sequenced as described (32). TCR nomenclature is in accordance with the International ImMunoGeneTics database (33).

Statistical analysis

All statistical analysis was performed using GraphPad Prism software (Graphpad Software Inc., San Diego, USA). The statistical significance between experimental groups was calculated using a Mann-Whitney test or a Kruskal-Wallis test followed by a Dunn's multiple comparison test. Results with a p value of less than 0.05 were considered significant.

Results

Detection of a $\gamma\delta$ T cell subset harboring a high level of CD3

We have recently demonstrated that $\gamma\delta$ T cells are an important early source of cytokines (IFN- γ and IL-17A) in response to α -GalCer (34). During our investigations, we have consistently observed in our control mice at steady-state, a minor population of hepatic $\gamma\delta$ T cells displaying an intense expression of CD3 compared to the remainder of the $\gamma\delta$ T cell population, but with a similar level of TCR expression (Fig. 1A). As shown in Figure 1B, this population is widely distributed, however in each organ tested, their frequency as a proportion of the whole $\gamma\delta$ T cell population differs. Thus, although this subset was preferentially represented in lungs, it was also detected in thymus and other peripheral organs, but not in the blood. Importantly, we were also able to detect these cells in BALB/c mice with a similar pattern of distribution to that observed in C57BL/6 (Figure S1). Moreover, their morphology was similar to normal lymphocytes (Fig. S1) and this population was absent in TCR $\delta^{-/-}$ mice (Fig. S1) reinforcing the idea that these cells are true $\gamma\delta$ T cells. Together our data define a population of $\gamma\delta$ T cells with a unique phenotype characterized by a bright expression of the CD3 complex.

CD3^{bright} $\gamma\delta$ T cells display a phenotype of $\gamma\delta$ T17 cells

Since no studies have yet reported a population of $\gamma\delta$ T cells with such a phenotype, we investigated the markers expressed by these cells in comparison to the conventional $\gamma\delta$ T cells. CD27 expression segregates $\gamma\delta$ T cell population into IFN- γ -producing (CD27⁺) and IL-17-producing (CD27⁻) $\gamma\delta$ T cells (35) and so represents a critical marker to define $\gamma\delta$ T cell functions. Strikingly, the CD3^{bright} cells uniformly lacked CD27 and NK1.1 expression, a phenotype of

$\gamma\delta$ T17 cells (Fig. 2A). A more extensive investigation of these cells determined that this population had an activated ($CD69^+$) (Fig. 2B) and effector memory phenotype ($CD62L^-$ $CD44^{high}$) (Fig. 2C) and was well equipped to produce T_H17 -related cytokines ($IL-1R1^+$ $IL-23R^+$ $IL-18R^+$) (Fig. 2D). Interestingly, we also detected the expression of receptors for soluble factors involved in leukocyte survival including IL-2 and IL-7, but not IL-15 (Fig. 2E). It is noteworthy that CD127 (IL-7R) and CD25 (IL-2R) expression on $CD3^{bright}$ $\gamma\delta$ T cells was higher compared to conventional $\gamma\delta$ T cells. $CD3^{bright}$ $\gamma\delta$ T cells also expressed the orphan nuclear receptor ROR γ t, a key transcription factor for IL-17 production (36, 37) (Fig. 2F) and the common marker of $\gamma\delta$ T cell-mediated cytotoxicity, NKG2D (Fig. 2G). Collectively, these data suggest that these cells have a phenotype of $\gamma\delta$ T17 cells.

$CD3^{bright}$ $\gamma\delta$ T cells express a canonical germline encoded V γ 6 /V δ 1 TCR

The nature of the TCR expressed by $\gamma\delta$ T cells has also been shown to correlate with their functions (38, 39). Here we have used the Heilig & Tonegawa nomenclature (40) and Elliott's nomenclature (41) to define the V γ and V δ chains respectively. Thus, expression of a V γ 1 $^+$ TCR is associated with a T_H1 -profile whereas V γ 4 $^+$ and V γ 6 $^+$ TCRs are mainly associated with a T_H17 phenotype (1). Given the limitation of commercially available monoclonal antibodies for specific γ and δ chain detection, we have used a single-cell RT-PCR multiplex system containing a set of primers for each gene encoding the different γ and δ chains (Fig. 3A). To date, all the $CD3^{bright}$ $\gamma\delta$ T cells were found to express a unique TCR δ rearrangement TRDV4-TRDD2-TRDJ2 encoding for a V δ 1-D δ 2-J δ 2 TCR (Fig. 3B). Of interest, the PCR products indicated that all V δ 1 $^+$ sequences included J δ 2 and had a similar CDR3 δ amino acid sequence with no N diversity. Study of the TCR γ repertoire also demonstrated a unique rearrangement TRGV6-TRGJ1

encoding for a V γ 6-J γ 1 TCR (Fig. 3B) with a similar CDR3 γ . By contrast, sequencing of the PCR products obtained with the conventional population indicated that this population expressed a more diverse TCR repertoire (Fig. 3B). These results suggest that CD3^{bright} $\gamma\delta$ T cells uniformly express a canonical fully germline encoded V γ 6V δ 1 TCR.

CD3^{bright} $\gamma\delta$ T cells are influenced by the endogenous flora and are ROR γ t-dependent.

According to their relative variable frequency between mice and their preferential distribution in mucosal sites, a place where immune cells are largely influenced by the microbiome, we have investigated the potential involvement of the commensal flora on the homeostasis of this particular subset of $\gamma\delta$ T cells. In line with our hypothesis, C57BL/6 mice bred in germ-free (GF) conditions had a selective reduction in their CD3^{bright} $\gamma\delta$ T cell compartment in the lungs, thymus, spleen, and liver compared to mice bred in SPF conditions (Fig. 4A). Moreover, mice chronically exposed to antibiotics (neomycin) also displayed a reduced frequency of CD3^{bright} $\gamma\delta$ T cells in the periphery (Fig. S2). By contrast, the frequency of conventional $\gamma\delta$ T cells was not modulated under these conditions. Interestingly the relationship to the microbiota seems to occur in a TLR-independent manner as MyD88^{-/-}TRIF^{-/-} mice show a normal CD3^{bright} $\gamma\delta$ T cell compartment (Fig. S2).

Expression of ROR γ t is mandatory in the development of T_H17 cells (36, 37) but appears dispensable for development and maintenance of $\gamma\delta$ T17 cells (9). However, using ROR γ t-deficient mice, we determined that CD3^{bright} $\gamma\delta$ T cells were fully dependent on the expression of ROR γ t (Fig. 4B). In addition, various cytokines including IL-6, IL-21, IL-1, and IL-23 have been demonstrated to be important in T_H17 cell differentiation and homeostasis (2). The frequency of

CD3^{bright} $\gamma\delta$ T cells was not modulated in mice lacking IL-23 (IL-23p19^{-/-} and IL-12p40^{-/-} mice) (Fig. 4C). Similarly, we were not able to detect a role for IL-1, IL-6 and IL-21 in the differentiation/maintenance of the CD3^{bright} $\gamma\delta$ T cell population (Fig. S2). The level of CD3 expression on these cells suggests an atypical CD3/TCR complex structure (42) with a perturbed stoichiometry, an observation already made for some $\gamma\delta$ T cell subsets (43). This could be due to an alternate structure with a biased expression of particular CD3 components such as the substitution of a CD3 ζ subunit by an Fc ϵ RI γ (44, 45). However, using Fc ϵ RI γ -deficient mice, we observed no modulation in the prevalence of CD3^{bright} $\gamma\delta$ T cells compared to control mice (Fig. S2).

CD3^{bright} $\gamma\delta$ T cell subset preferentially produces IL-17A upon activation

Having established that this CD3^{bright} $\gamma\delta$ T cell population displayed a phenotype of IL-17-producing cells, we next checked whether indeed these cells produced IL-17. To investigate this, we purified $\gamma\delta$ T cells from lungs according to their CD3 expression and subjected them to PMA/ionomycin stimulation. As depicted in Figure 5A, this resulted in a large secretion of cytokines by $\gamma\delta$ T cell subsets including IFN- γ and IL-17A. Interestingly, the two subsets displayed a clear dichotomy in their cytokine profile. The conventional $\gamma\delta$ T cells showed a preferential T_H1-bias while the CD3^{bright} subset was only capable of secreting IL-17A. Of note, this was observed using both hepatic and splenic CD3^{bright} $\gamma\delta$ T cells (Fig. S3). Recently, CD27 has been demonstrated to be a thymic determinant for $\gamma\delta$ T cell differentiation where the CD27⁻ subset was associated with a T_H17 profile (35). Therefore we investigated in our *in vitro* assay whether the CD3^{bright} $\gamma\delta$ T cell subset (CD27⁻ NK1.1⁻) had a distinct ability to secrete IL-17A compared to the CD27⁻ subset represented in the conventional population of $\gamma\delta$ T cells (Fig. S3).

In line with the study from Ribot et al. (35) we observed that CD27⁺ $\gamma\delta$ T cells produced IFN- γ , but no IL-17A, whereas CD27⁻ $\gamma\delta$ T cells secreted IL-17A, but no IFN- γ . Importantly however, comparison of conventional CD27⁻ $\gamma\delta$ T cells *vs* CD3^{bright} $\gamma\delta$ T cells indicated that the latter were more capable of producing IL-17A (Fig. S3). We also did a similar analysis to compare CD3^{bright} $\gamma\delta$ T cells and V γ 4⁺ $\gamma\delta$ T cells, another TCR chain strongly associated with IL-17 production (39). Again, CD3^{bright} $\gamma\delta$ T cells produced more IL-17A than V γ 4⁺ $\gamma\delta$ T cells in this setting (Fig. S3). Furthermore, despite a clear ability to produce IL-17, V γ 4⁺ $\gamma\delta$ T cells were also able to secrete a substantial amount of IFN- γ . In contrast, activation of CD3^{bright} $\gamma\delta$ T cells by means of anti-CD3 stimulation also resulted in IL-17A production, but not IFN- γ (Fig. 5B). Together, these data demonstrate that the CD3^{bright} $\gamma\delta$ T cells are intrinsically skewed towards an IL-17A-producing cell profile and have a higher ability to produce this cytokine compared to other $\gamma\delta$ T cells.

IL-23 and NLRP3 inflammasome-dependent IL-1 β are required for IL-17A production by CD3^{bright} $\gamma\delta$ T cells

We have recently demonstrated that α -GalCer injection resulted in a rapid production of IL-17A by $\gamma\delta$ T cells (34). According to the hallmarks of CD3^{bright} $\gamma\delta$ T cells, this population represented an interesting candidate to be a preferential IL-17-producing $\gamma\delta$ T cell subset in response to α -GalCer. In line with our hypothesis, we observed that hepatic CD3^{bright} subsets of $\gamma\delta$ T cells rapidly produced IL-17A after α -GalCer administration (Fig. 6A). In stark contrast, only few conventional $\gamma\delta$ T cells (despite a significant proportion of CD27⁻ $\gamma\delta$ T cells) produced IL-17A (Fig. 6A). Interestingly, we found that a large proportion of CD3^{bright} $\gamma\delta$ T cells (~ 50%) produced IL-17A only 2 hrs post- α -GalCer injection and partially sustained this secretion for another 10

hrs. This subset did not secrete IFN- γ at any time studied, but in concert with our recent report (34), non-CD3^{bright} $\gamma\delta$ T cells were capable of secreting a substantial amount of IFN- γ (Fig. 6A). We next investigated whether the CD3^{bright} $\gamma\delta$ T cells were also capable of producing IL-17A in an immune response to LPS. As expected, $\gamma\delta$ T cells were an important source of IL-17A (46), but only ~11% of conventional $\gamma\delta$ T cells produced IL-17A in response to LPS, whereas 83% CD3^{bright} $\gamma\delta$ T cells secreted this cytokine (Fig. 6B).

Recent studies have suggested that IL-1 β and IL-23 drive IL-17A secretion by $\gamma\delta$ T cells in the absence of TCR ligation (27, 29). To test this hypothesis, we have used IL-1R1^{-/-} mice or WT mice pre-treated with an anti-IL-23p19 mAb. Interestingly, we observed that IL-17A production by hepatic CD3^{bright} $\gamma\delta$ T cells was significantly impaired in both settings (Fig. 6C). As expected, the employment of gene-targeted mice for NLRP3-inflammasome components (NLRP3 and caspase-1) indicated that IL-1 β -dependent IL-17A production was dependent on the NLRP3/caspase-1 activation cascade (Fig. S4). Experiments conducted with LPS indicated a similar mechanism of activation (Fig. 6D). To further confirm these findings, we checked if these cytokines were sufficient to induce IL-17A production by highly purified $\gamma\delta$ T cells. In agreement with our recent report and others (27, 29, 34), combination of IL-12 and IL-18 led to IFN- γ production by conventional $\gamma\delta$ T cells whereas IL-23 plus IL-1 β resulted in IL-17A secretion (Figure S4). The same combinations incubated with CD3^{bright} $\gamma\delta$ T cells also resulted in the secretion of IL-17A (IL-23 + IL-1 β), but not IFN- γ (IL-12 + IL-18) (Fig. S4). Notably, at the doses tested, individual recombinant cytokines failed to promote cytokine production. Together these data indicate that IL-23 and IL-1 β are important for IL-17A production by CD3^{bright} $\gamma\delta$ T

cells in response to α -GalCer and suggest that these cytokines are sufficient to induce IL-17A production by purified CD3^{bright} $\gamma\delta$ T cells.

CD3^{bright} $\gamma\delta$ T cells display a feature of specialized cells against pulmonary pathogens.

With respect to the preferential localization in lungs and the ability of CD3^{bright} $\gamma\delta$ T cells to rapidly produce large amount of IL-17A, we next investigated their potential biological activity in lungs. Intranasal injection of α -GalCer, the prototypical type 1 NKT cell activator induces airway neutrophilia in an IL-17-dependent mechanism (30, 47, 48) and its activity as a mucosal adjuvant is now widely accepted for induction of protective immunity against pathogens (30, 49, 50). Intranasal instillation of α -GalCer resulted in IL-17A and IFN- γ production by $\gamma\delta$ T and type I NKT cells (Fig. 7A and Fig. S5). Notably, the CD3^{bright} $\gamma\delta$ T cells were prominent producers of IL-17A on a per cell basis compared to conventional $\gamma\delta$ T cells and type I NKT cells (Fig. 7A). As expected, this was accompanied by airway neutrophilia 8 hrs after α -GalCer instillation (Fig. 7B); an observation largely dependent on $\gamma\delta$ T cells (Fig. 7B) suggesting that the CD3^{bright} $\gamma\delta$ T cells might participate in this effect. As observed in the liver after systemic administration of α -GalCer, the pool of CD3^{bright} $\gamma\delta$ T cells in lungs expanded by ~ 10 fold within 24 hrs of i.n. administration of α -GalCer (Fig. 7C). Using a relevant model of respiratory bacterial infection, we observed that CD3^{bright} $\gamma\delta$ T cells were early and major sources of IL-17 following intranasal *Streptococcus pneumoniae* infection (Fig. 7D). As expected, unlike the conventional subset, CD3^{bright} $\gamma\delta$ T cells failed to secrete IFN- γ (Fig. S5). Recently expression of CCR6 has been correlated with $\gamma\delta$ T17 cells (51). Although IL-17 production was restricted to the CCR6⁺ subset in conventional $\gamma\delta$ T cells, CD3^{bright} $\gamma\delta$ T cells could produce IL-17 independently from CCR6

expression (Fig. S6). Moreover, similar to the α -GalCer model, the frequency and number of these cells selectively increased during the early course of infection (Fig. 7E). Altogether these data indicate that CD3^{bright} $\gamma\delta$ T cells may play a critical role in mucosal immunity against respiratory pathogens.

Discussion

The detection and separation of immune cells with specialized functions is important as this offers more refined targets for immunotherapeutic purposes. Here, we have reported a distinct subset of $\gamma\delta$ T cells with a heightened ability to produce IL-17A based on their high expression of CD3. CD3^{bright} $\gamma\delta$ T cells are a homogenous population predominantly represented in lungs that display a phenotype of effector memory IL-17-producing cells and in different mouse backgrounds (C57BL/6 and BALB/c). These cells use a canonical germline encoded V γ 6V δ 1 TCR. All previous reports on V γ 6V δ 1 $\gamma\delta$ T cells have used PCR-based strategies or a monoclonal Ab recognizing all V δ 1⁺ $\gamma\delta$ T cells (including V γ 5/V δ 1 $\gamma\delta$ T cells) (42, 52). Here we describe a simple gating strategy to specifically analyze V γ 6V δ 1 $\gamma\delta$ T cells using CD3. This new strategy allowed us to determine new features about their phenotype and factors regulating their development/homeostasis.

Of interest, breeding mice in a germ-free environment profoundly affected the CD3^{bright} $\gamma\delta$ T cell compartment in the periphery indicating that this subset is tightly regulated by commensal bacteria. Interestingly this phenotype is recapitulated after prolonged exposure of SPF adult mice to antibiotics suggesting that the microbiome plays a role on expansion/maintenance of these cells rather than their development. This finding is in concert with a recent study demonstrating that some specific commensals are required to maintain IL-1R1⁺ $\gamma\delta$ T cells through a signaling pathway involving the proto-oncogene Vav1 (53). Given the potential role of this population in antimicrobial immunity (9, 21, 52), this observation is in line with the consensus that chronic exposure to antibiotics results in transient immunodeficiency and increased susceptibility to (super)-infections (54).

How microbiota impacts on CD3^{bright} $\gamma\delta$ T cells remains unclear, although these organisms appear to promote IL-7 production under homeostatic conditions (55), a factor important to maintain ROR γ t expression in innate lymphocytes (56). Interestingly, we have demonstrated that CD3^{bright} $\gamma\delta$ T cells are completely dependent on ROR γ t expression for their development and maintenance in the periphery. Moreover, these cells have a bright expression of IL-7R compared to other leukocytes and, in line with a recent report (28) and our unpublished data, this population promptly responds to IL-7, which may be a selective growth factor for $\gamma\delta$ T17 cells (28). Since Notch1 signaling triggers CD127 expression and IL-7 signaling (57, 58), the intense expression of this receptor may result from the involvement of Notch1 during the development of $\gamma\delta$ T17 cells (9) and therefore, based on our findings, CD3^{bright} $\gamma\delta$ T cells. However in the same study, the authors demonstrated that ROR γ t expression was dispensable for the development and maintenance of $\gamma\delta$ T17 cells. The reason for this discrepancy is likely to be due to the fact that we focused on the CD3^{bright} $\gamma\delta$ T subset while the previous report analyzed the entire pool of $\gamma\delta$ T17 cells that may contain both ROR γ t-dependent (innate V γ 6⁺ cells) and -independent (acquired V γ 4⁺ cells) $\gamma\delta$ T cells. Consistent with this idea, $\gamma\delta$ T17 cells (CD27⁻) from the conventional population were only minimally affected by the absence of ROR γ t expression (not shown).

The level of CD3 expression is also intriguing and suggests that these cells may have a unique CD3/TCR complex. Consistent with this observation, a previous report had demonstrated that the 17D1 mAb, which primarily detects V γ 5V δ 1 TCR, can cross-react with V γ 6V δ 1 TCR if the structure is first stabilized with an anti-C δ mAb suggesting an alternate structure for this CD3/TCR complex (42). Thus, the differential CD3 expression between the V γ 6V δ 1 TCR and the other $\gamma\delta$ TCRs could explain this observation. Further biochemical analysis will help to

examine the precise structure of the CD3 complex and investigate if this feature could partially explain their particular biology. Nevertheless, an alternate structure involving FcεRIγ (44, 45) cannot be considered as CD3^{bright} γδ T cells from mice deficient for this signaling chain have a normal distribution pattern. Furthermore, the canonical germline encoded Vγ6Vδ1 TCR expressed with the high level of CD3 expressed suggests that this TCR does not engage any recombination processes or positive selection during ontogeny. This observation is important regarding the current controversial hypotheses concerning thymic development of γδT17 cells. While studies suggest that γδT17 arise from thymus during embryonic life and persist in adults through self-renewal process (9, 19, 35), the requirement of TCR ligation in their development is still debated (22, 24, 59). A recent study elegantly demonstrated that γδT17 can produce IL-17 in the embryonic thymus prior to TCR rearrangement suggesting that these cells may be selected in a TCR-independent manner (19). By contrast, a previous report suggested that γδT17 selection might require a weak TCR engagement (24). Overall, our data mainly support the first hypothesis and emphasize the innate imprint of the CD3^{bright} γδT cells.

There remains a critical question about the real function of the TCRs expressed on γδT17 cells, especially on Vγ6Vδ1 T cells (60). It seems clear that TCR engagement by anti-CD3 antibody resulted in cytokine release (IL-17A and IL-22), indicating that this TCR is functional. While soluble factors like IL-1β and IL-23 appear sufficient to induce IL-17A production by highly purified Vγ6Vδ1 T cells, we cannot exclude the possibility that the anti-TCRδ and anti-CD3 mAbs used to sort these cells may contribute to their cytokine production via TCR engagement mediated activation. It is also possible that their TCR engages antigens expressed by the same adjacent cells in the culture. Interestingly, using the same number of cells, CD3^{bright} γδT cells

were more sensitive to CD3 triggering compared to conventional $\gamma\delta$ and $\alpha\beta$ T cells (Figure 5 and not shown). While the higher level of CD3 expressed by this subset is the simplest explanation for this hyperreactivity, it may also reflect their memory phenotype which may render them more sensitive to TCR signaling pathway (61). However, further studies will be required to understand if this TCR should be considered as a conventional TCR that recognize Ag/Ag-presenting molecule complex or more as a pattern recognition receptor, a homing receptor, or a vestigial structure that may be simply required for the development/expansion of these cells.

Using various activation strategies (TCR-dependent and -independent), we consistently observed that V γ 6V δ 1 could only produce T_H17-related cytokines, indicating a fully differentiated subset with a pre-programmed capacity to produce this cytokine. This specialized function suggests a selection pressure to maintain this very population of innate cells throughout evolution and is in line with their memory phenotype. To our best knowledge, it is the first time that an entire population of cells carrying a canonical TCR produces such a conserved spectrum of cytokines to various stimuli. This pool of cells can either be rapidly expanded and/or recruited at sites of inflammation, which could compensate for their low number at steady-state. The cytotoxic capacity of these cells has not been directly investigated in our study, but it is noteworthy that CD3^{bright} $\gamma\delta$ T cells displayed a high expression of NKG2D at steady-state, which might imply potential capacity to mediate cytotoxicity on transformed or virus-infected cells.

Interestingly we have observed in human a population displaying a similar bright expression of CD3 and phenotypically associated with a T_H17 cell signature. However further studies are required to understand if these populations can be somehow related. Such a classification across

species would represent a significant step in understanding the functional dichotomy of $\gamma\delta$ T cell subsets.

The presence of V γ 6V δ 1 T cells in various organs has been demonstrated during early life (42). Thus it is possible that V γ 6V δ 1 T cells play a major role during early life, at a time the adaptive immune system remains not fully educated. In any case, the clonality of this population at this stage of life has never been studied and the preferential persistence of the CD3^{bright} $\gamma\delta$ T cells in particular niches in adults might be due to the presence of a favorable local environment (e.g. self-Ag, cytokines, and particular homing receptors) for these cells. Nonetheless, the selection pressure which conserved this population in different backgrounds may also imply specialized functions in some organs. In line with this, the rapid response of CD3^{bright} $\gamma\delta$ T cells after microbial insults is astounding and indicates that this population is likely to be involved in protection against pathogens especially at mucosal sites. Given that CD3^{bright} $\gamma\delta$ T cells represent the major sources of T_H17 cytokines during the early course of *S. pneumoniae* infection, it is now important to investigate the functions of these cells in this context and more broadly during various pulmonary disorders. Accordingly, we have identified a subset of $\gamma\delta$ T cells with a potent ability to produce IL-17 and with putative key functions in a broad range of IL-17-driven pathologies.

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Figure legends

Figure 1: Distribution of CD3^{bright} $\gamma\delta$ T cells. *A*, hepatic cells from naïve C57BL/6 WT mice were analyzed by flow cytometry based on CD3 and TCR $\gamma\delta$ expression. *B*, cells from lungs, thymus, spleen, lymph nodes, bone marrow and peripheral blood were analyzed as in panel *A* (*upper panel*). The mean percentage \pm SEM of CD3^{bright} $\gamma\delta$ T cells in total $\gamma\delta$ T cells from various organs of three independent experiments (3-5 mice/experiment) is shown in the *lower panel* ND, not detected.

Figure 2: Phenotype of pulmonary CD3^{bright} $\gamma\delta$ T cells vs conventional $\gamma\delta$ T cells. Pulmonary $\gamma\delta$ T cell subsets from naïve C57BL/6 WT mice have been stained with different combinations of antibodies and were analyzed for CD27 and NK1.1 (*A*), CD69 (*B*), CD62L and CD44 (*C*), IL-23R, CD121a and IL-18R (*D*), CD25, CD122 and CD127 (*E*), ROR γ t (*F*) and NKG2D (*G*) expression. Of note, expression of IL-23R (*D*) and ROR γ t (*F*) were assessed by the use of reporter mice using IL-23R^{+/-}-GFP knock-in and ROR γ t^{+/-}-GFP knock-in mice, respectively. The histogram in panel *C* (*right panel*) represents the differential CD44 expression between CD3^{bright} and conventional $\gamma\delta$ T cell subsets. *D*, *E* and *G*, line histograms demonstrate expression of each marker and filled histograms show their respective isotype controls. Numbers indicated in dot plots/histograms represent either mean of fluorescence intensity (MFI) or percentage of positive cells for the marker tested from at least 3 independent experiments (4-5 mice/experiment).

Figure 3: CD3^{bright} $\gamma\delta$ T cells have an invariant TCR. *A*, PCR analysis of cDNA isolated from single cell-sorted CD3^{bright} and conventional $\gamma\delta$ T cells and amplified with a set of primers specific for each TCR γ V-gene segment or the corresponding constant region (C γ) (upper row) or specific for each TCR δ V-gene segment or the corresponding constant region (C δ) (lower row). Clones have been sorted from two independent experiments using a cell suspension of a pool of at least 5 mice. *B*, Sequences for CD3^{bright} and conventional $\gamma\delta$ TCRs including diversity (D) (for δ chains), junction (J) and CDR3 region.

Figure 4: Factors involved in CD3^{bright} $\gamma\delta$ T cell homeostasis. *A*, frequency of $\gamma\delta$ T cell subsets based on CD3 expression was analyzed by flow cytometry in mice bred either in GF or SPF conditions. The percentage of $\gamma\delta$ T cell subsets is shown (*upper panel*). The average \pm SEM of $\gamma\delta$ T cell subset frequencies pooled from two independent experiments (3-6 mice/group/experiment) is shown in the *lower panel*. *, $p < 0.05$ and **, $p < 0.01$. Of note, the total number of cells in each organ was not different between GF and SPF mice. *B*, frequency of $\gamma\delta$ T cell subsets was analyzed by flow cytometry in WT vs ROR γ t^{-/-} mice. The percentage of $\gamma\delta$ T cell subsets is shown (*left panel*). The mean \pm SEM of $\gamma\delta$ T cell subset frequencies pooled from two independent experiments is shown in the *right panel* (4 mice/group/experiment) **, $p < 0.01$ and ***, $p < 0.001$. *C*, frequency of $\gamma\delta$ T cell subsets was analyzed by flow cytometry in WT vs IL-23p19^{-/-} mice. The percentage of $\gamma\delta$ T cell subsets is shown (*left panel*). The mean \pm SEM of $\gamma\delta$ T cell subset frequencies of one representative experiment out of two is shown in the *right panel* (5 mice/group/experiment).

Figure 5: Pulmonary CD3^{bright} $\gamma\delta$ T cells produce IL-17A *in vitro*. FACS-sorted pulmonary $\gamma\delta$ T cell subsets and NK cells (positive control) were incubated with PMA (10 ng/ml) and ionomycin (1 μ g/ml) (A) or pre-coated anti-CD3 (10, 5 and 1 μ g/ml) (B) for 20 hours. Cytokine production in the supernatants was measured by CBA. Data represent the mean \pm SEM of two pooled independent experiments performed in duplicate.

Figure 6: Hepatic CD3^{bright} $\gamma\delta$ T cells preferentially produce IL-17A *in vivo* after α -GalCer or LPS administration. A, WT mice were injected i.p. with vehicle or α -GalCer (2 μ g/mouse) and were killed at indicated time points. Gated hepatic $\gamma\delta$ T cell subsets were screened for IFN- γ and IL-17A production. One representative experiment out of three is shown (*upper panel*). Gates were set based on the staining with isotype control. The pooled mean \pm SEM of three independent experiments for IFN- γ - and IL-17A-positive $\gamma\delta$ T cell subsets is shown in the *lower panel* (3-4 mice/group/experiment). ***, $p < 0.001$. Total number of hepatic $\gamma\delta$ T cell subsets was assessed. ***, $p < 0.001$. B, WT mice were injected i.p. with PBS or LPS (0.5 mg/mouse) and were sacrificed after 8 hrs. Liver cells were treated as in panel A. One representative experiment out of two is shown (*upper panel*). Gates were set based on the staining with isotype control. The mean \pm SEM of two independent experiments for IL-17A-positive $\gamma\delta$ T cell subsets is shown in the *lower panel* (3 mice/group/experiment). C, WT mice were pre-treated with an anti-IL23p19 or isotype control (agp3) (500 μ g/mouse) at day -1 and 0 and then injected i.p. with vehicle or α -GalCer (2 μ g/mouse) (*left panels*). WT or IL-1R1^{-/-} mice were injected i.p. with vehicle or α -GalCer (2 μ g/mouse) (*right panels*). Mice were sacrificed 2 hours after α -GalCer administration. Liver cells were treated as in panel A. The mean \pm SEM of two pooled independent experiments for IL-17A-positive CD3^{bright} $\gamma\delta$ T cells is shown (4 mice/group/ experiment) ***, $p < 0.001$. D,

IL-23-depleted or controls WT mice were injected with LPS as in panel B. The mean \pm SEM of two independent experiments for IL-17A-positive $\gamma\delta$ T cell subsets is shown (3 mice/group/experiment). *, $p < 0.05$ and **, $p < 0.01$.

Figure 7: Pulmonary $\gamma\delta$ T cells produce IL-17A *in vivo* after i.n. α -GalCer instillation and contribute to airway neutrophilia. A-C, WT mice were administered i.n. with vehicle or α -GalCer (500 ng/mouse) and were sacrificed at indicated time points. A, Gated pulmonary $\gamma\delta$ T cell subsets were screened for IFN- γ and IL-17A production. One representative experiment out of three is shown (*upper panel*). Gates were set based on the staining with isotype control. The mean \pm SEM for IFN- γ - and IL-17A-positive $\gamma\delta$ T cell subsets is shown in the *lower panel* (6 mice/group/experiment). ***, $p < 0.001$. B, WT and TCR $\delta^{-/-}$ mice were injected i.n. with vehicle or α -GalCer and were sacrificed after 8 hours. The total number of cells in BAL was determined and differential proportion of leukocytes was counted under the microscope. One experiment out of two is shown ($n = 7$). *, $p < 0.05$; **, $p < 0.01$. C, WT mice were injected i.n. with vehicle or α -GalCer and were sacrificed after 24 hours. The frequency and total number of $\gamma\delta$ T cell populations in the lungs was determined by flow cytometry. One experiment out of two is shown ($n = 7$). **, $p < 0.01$. D and E, WT mice were infected i.n. with *Streptococcus pneumoniae* serotype 1 (2×10^6 bacteria) and were sacrificed at indicated time points. D, Gated pulmonary $\gamma\delta$ T cell subsets were screened for IL-17A production. One representative experiment out of three is shown (*upper panel*). Gates were set based on the staining with isotype control. The mean and absolute number \pm SEM for IL-17A-positive $\gamma\delta$ T cell subsets is shown in the *lower panel* (>3 mice/group/experiment) *, $p < 0.05$ and **, $p < 0.01$. E, Percentage of $\gamma\delta$ T cell subsets is shown

in the upper panel. Absolute number of IL-17A-positive $\gamma\delta$ T cell subsets is shown in the lower panel. *, $p < 0.05$.

Table 1: Primers for mouse TCR γ and TCR δ chains

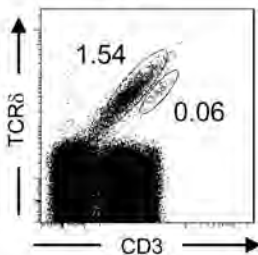
IMGT	V γ	External	Internal
TRGV1	1	CGGTCACCAGAGMGACAGATG	ATGTCTCAACAACTACAATCAACG
TRGV2	2	CGGTCACCAGASAGACAGATG	ATGTCGCAACAACTACAATCAACG
TRGV3	3	CGGCAAAAACCAAATCAACAGT	CTACAATCAACRACCCCTTAGGAG
TRGV4	4	GCAACCCCTACCCATRTTTTCT	GGAGTACAAGAAAATGGARGCAA
TRGV5	5	CCCGAAGACCAAACAAGACG	CCCTGAGACGAATCTTCTATGG
TRGV6	6	AAGTGTTTCAGAAGCCCGATG	GACGAAAGATATGAGGCAAGGA
TRGV7	7	AATGTCAATCACCAAGCTAGAGG	TGCTTCGGAATGTGGAGGAG
TRGC Rev1	-	TGACAATACATCTGTGCTCTTTCC	GCAGCAGAAGGAAGGAAAATAGTA
TRGC Rev2	-	TGACAATGCAACTATGTTCTTTAGC	GCAACAGAAGGAAGRAAAATAGTG
	V δ	External	Internal
TRDV4	1	CTTCTACTGCACTGTAACAGGAGG	CTCGACATTGAGAAGGCAACA
TRDV1	2	GAGCTGCAATTTATCGACATCTT	GGTATAGGCAGGGGGATGAT
TRDV2-2	4	GGACTGCACCTATGACACAAGTGA	TCKCCAAGAAGCATACAAGC

TRDV5	5	GTACAATGCGGATTCTCCAAAC	TATCGCAAAAGGCCAGACAG
TRDC Rev	-	GCACTGTACTTCCCGCTGG	ATCTTTCACCAGACAAGCAACA

Figure 1

A

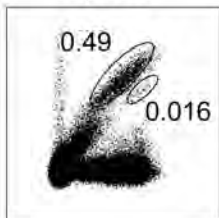
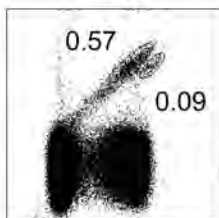
Liver



B

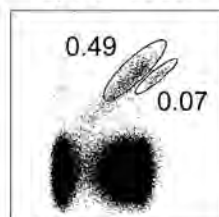
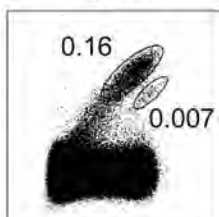
Lungs

Spleen



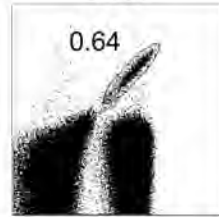
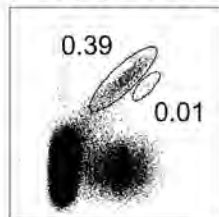
Thymus

Lymph node



Bone marrow

Blood



Distribution of CD3^{bright} $\gamma\delta$ T cells

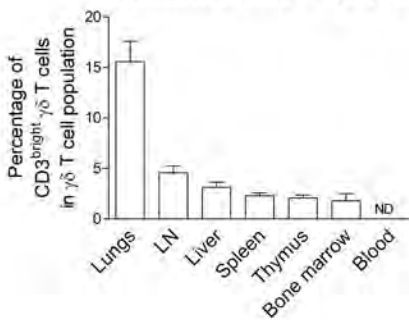
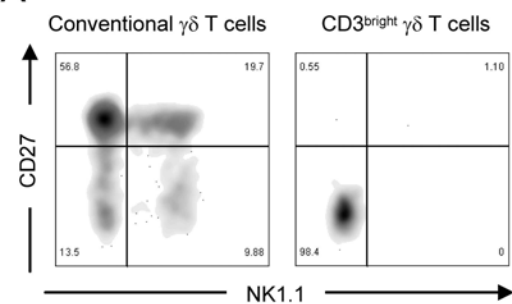
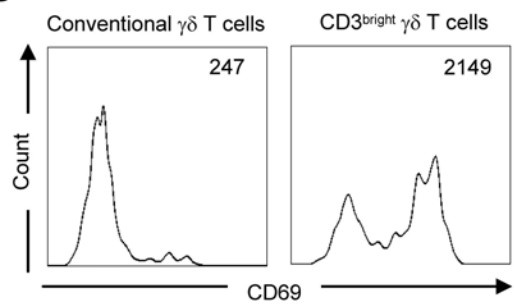


Figure 2

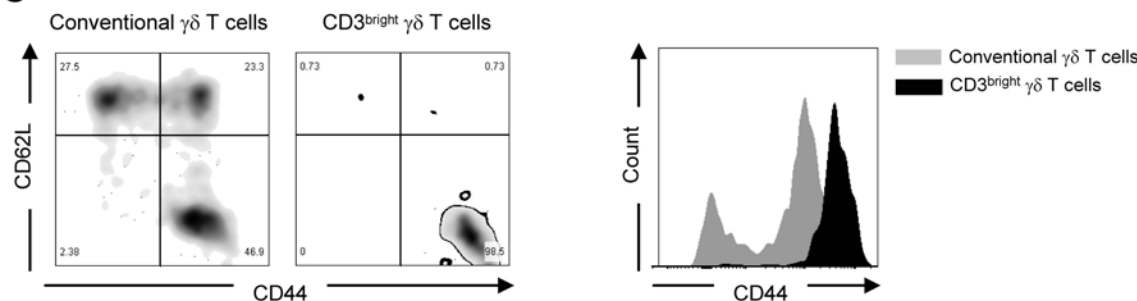
A



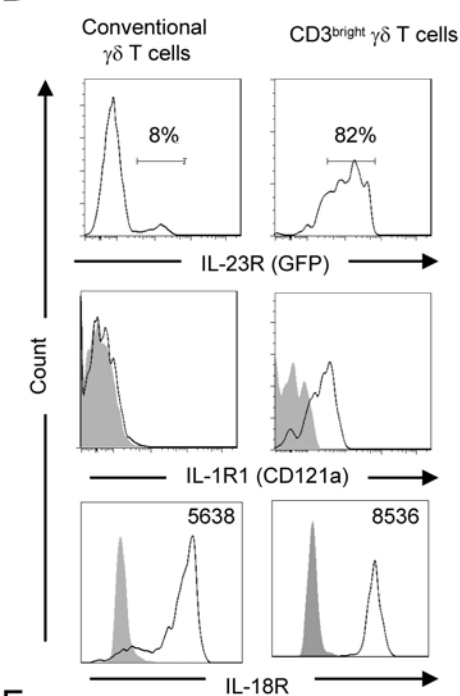
B



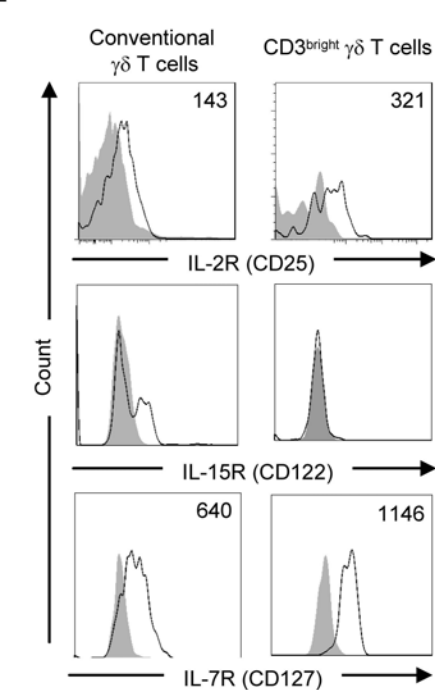
C



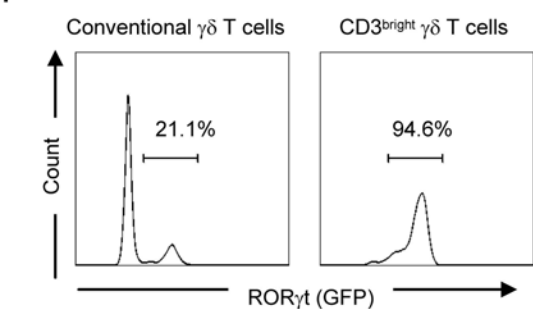
D



E



F



G

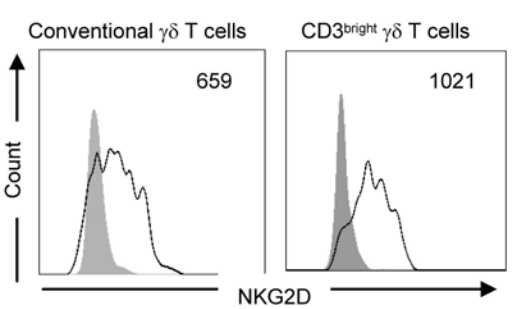
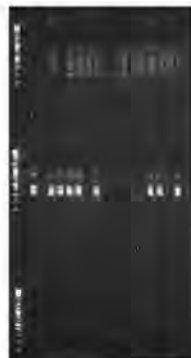


Figure 3

A

TRGV

TRDV



CD3^{bright} γδT cells

Conventional γδT cells

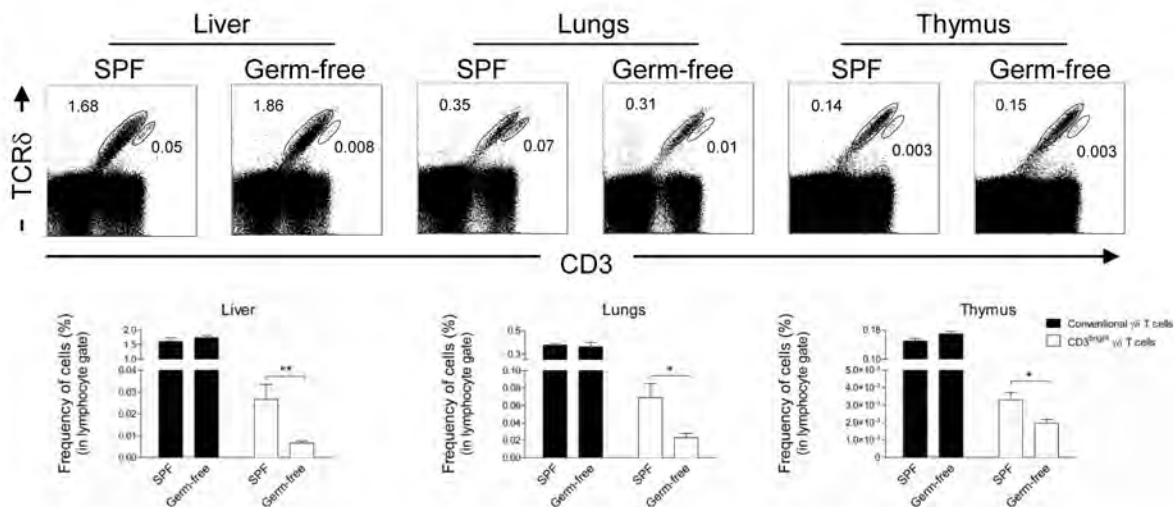
Negative controls

B

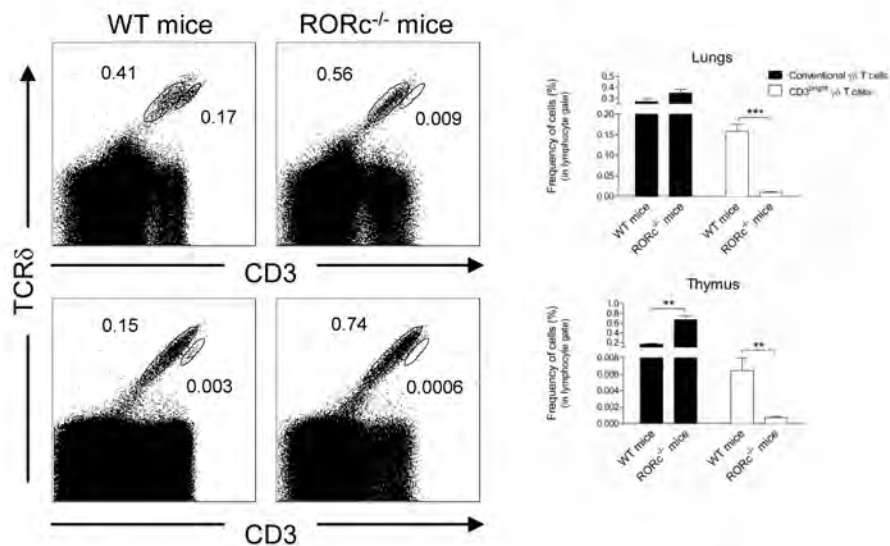
Subsets	Clones	V _γ	CDR3 _γ	J _γ	V _δ	CDR3 _δ	D _δ	J _δ
Conventional γδT cells	1	2	CAVIMRYSSGFHKVF	2	5	CASGYSWIHGGIRSSSTDH LVF	2	1
	2	1/4	-	-	5	CASGYVYGTLRYRDTDKLVF	1	1
	3	1/4	-	-	4	CALMERGAFFPYRDKGTDKLVF	1	1
	4	1/4	-	-	5	CASGYIGGIRATDKLVF	2	1
	5	2	CAVCRLARKF#KVF	2	2	CALMLYGLLSEGPTTDKLVF	1	1
	6	2	CAVRISSGFHKVF	2	5	CASGPIGGIRATDKLVF	1	1
	7	2	CAVLDSGFFHKVF	2	5	CASGYIGGIRATDKLVF	1	1
	8	2 sequences - undecipherable			5	CASGYIGGIRATDKLVF	1	1
	9	2 sequences - undecipherable			5	CASGHWGYETDKLVF	1	1
CD3 ^{bright} γδT cells	1	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	2	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	3	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	4	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	5	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	6	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	7	6	CACWDSSGFCHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	8	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	9	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	10	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	11	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2
	12	6	CACWDSSGFHKVF	1	1	CQSDIGGSSWDTRQMFF	2	2

Figure 4

A



B



C

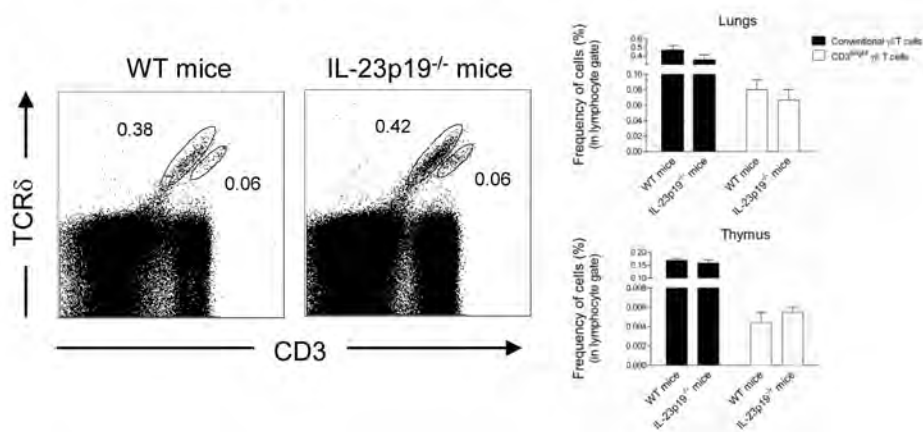
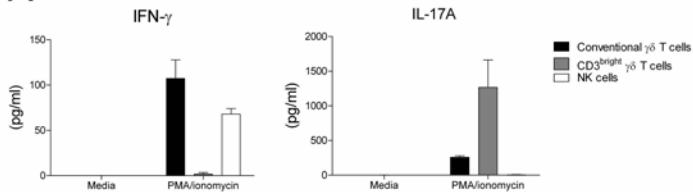


Figure 5

A



B

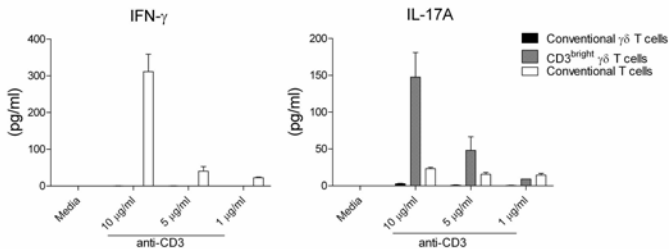
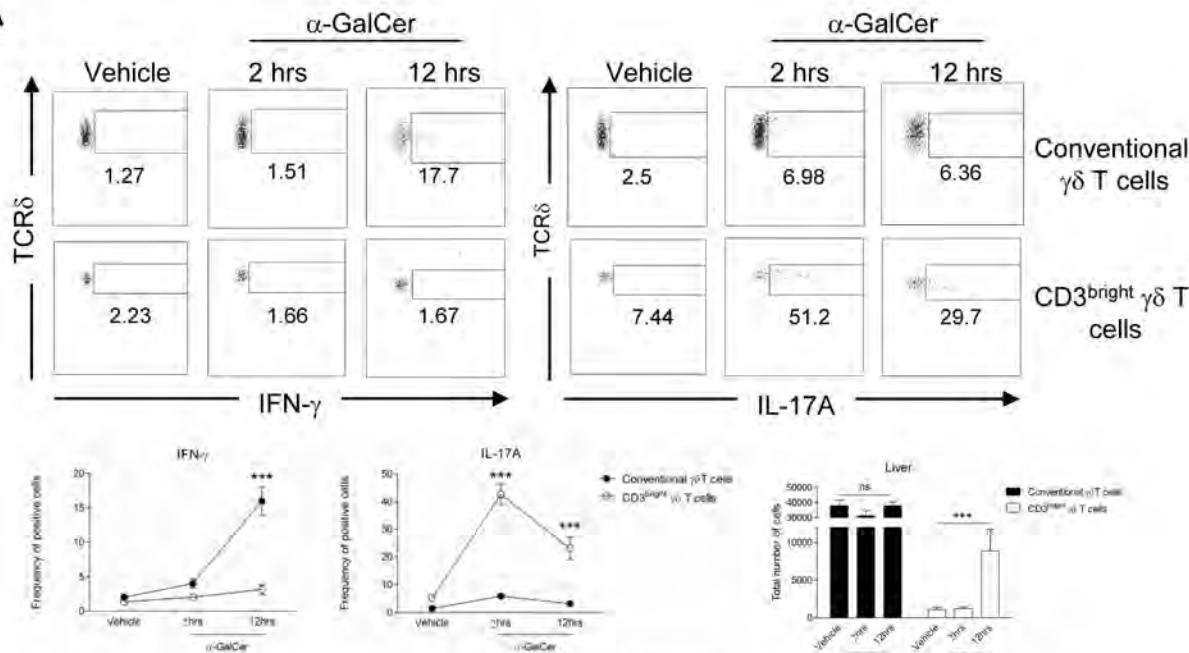
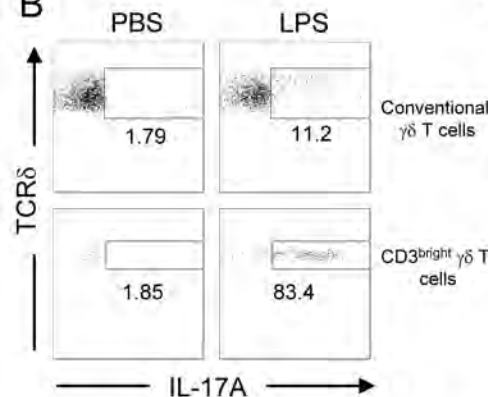


Figure 6

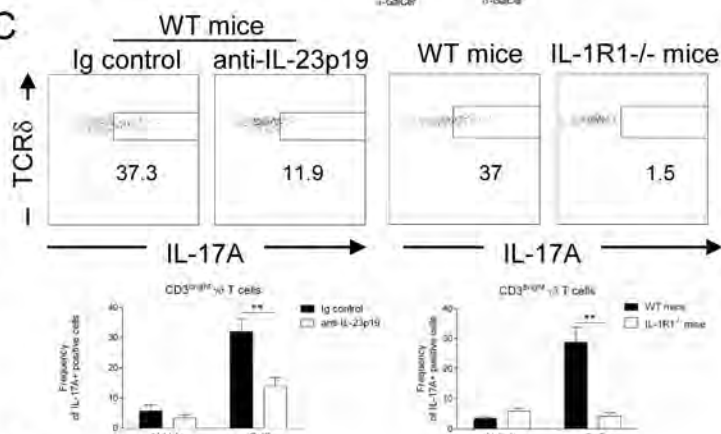
A



B



C



D

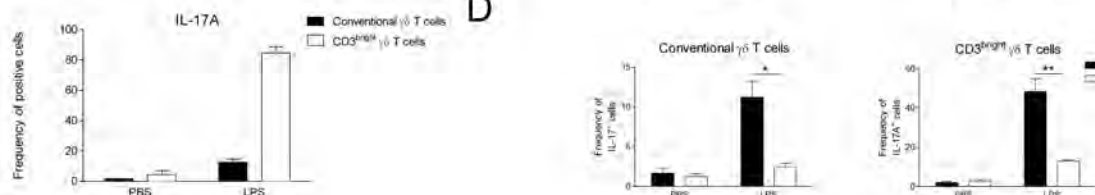
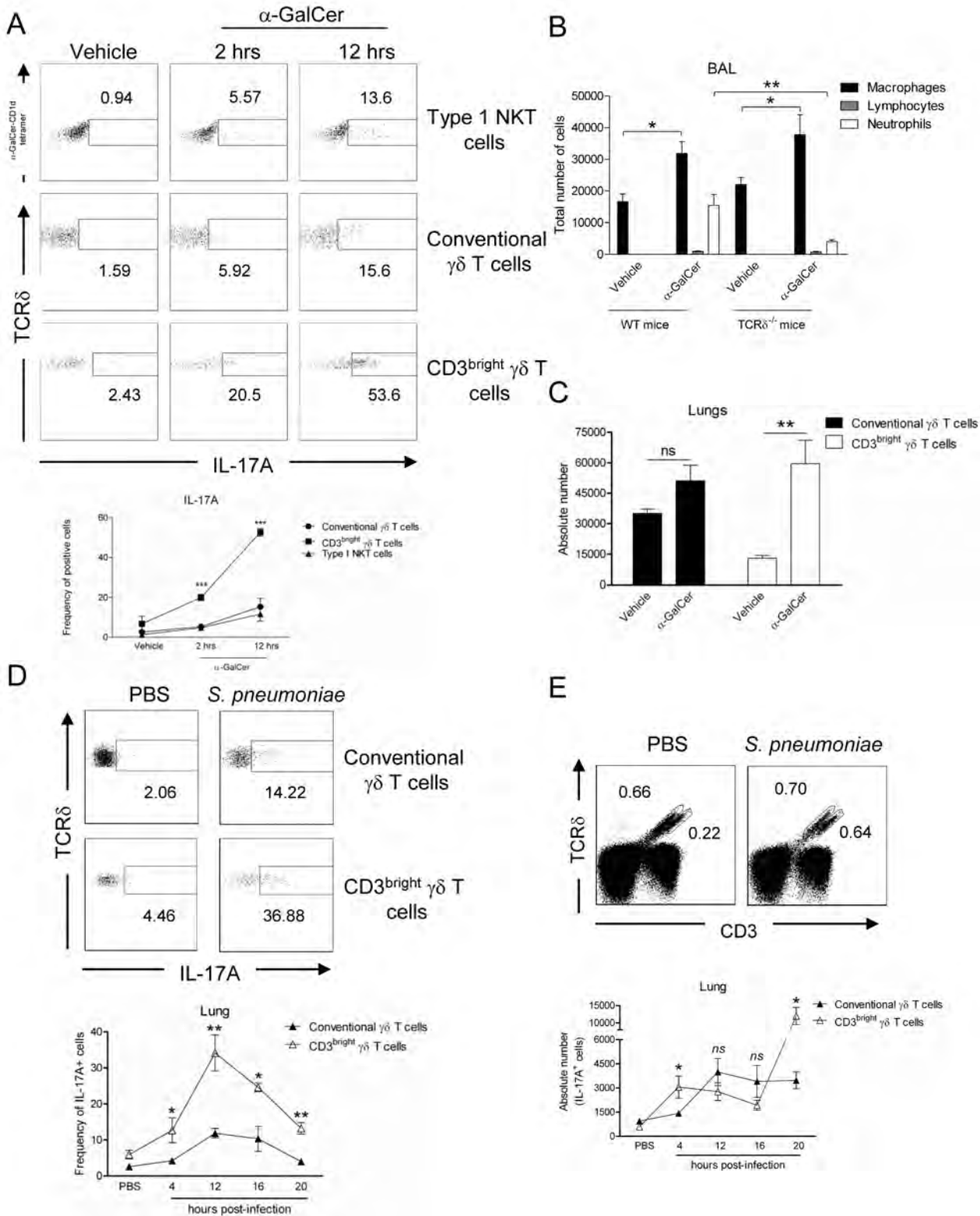


Figure 7



Supplemental information:

CD3 expression reveals a gamma/delta T cell subset with specialized functions

Christophe Paget, Melvyn T. Chow, Nicholas A. Gherardin, Paul A. Beavis, Adam P. Uldrich, Helene Duret, Maya Hassane, Fernando S.F. Guimares, Goeffrey R. Hill, Paul Neeson, David S. Ritchie, François Trottein, Gabrielle T. Belz, Dale I. Godfrey and Mark J. Smyth

Supplemental Inventory:

Figure S1, related to Figure 1
Figure S2, related to Figure 4
Figure S3, related to Figure 5
Figure S4, related to Figure 6
Figure S5, related to Figure 7
Figure S6, related to Figure 7

Supplemental Figures:

Figure S1

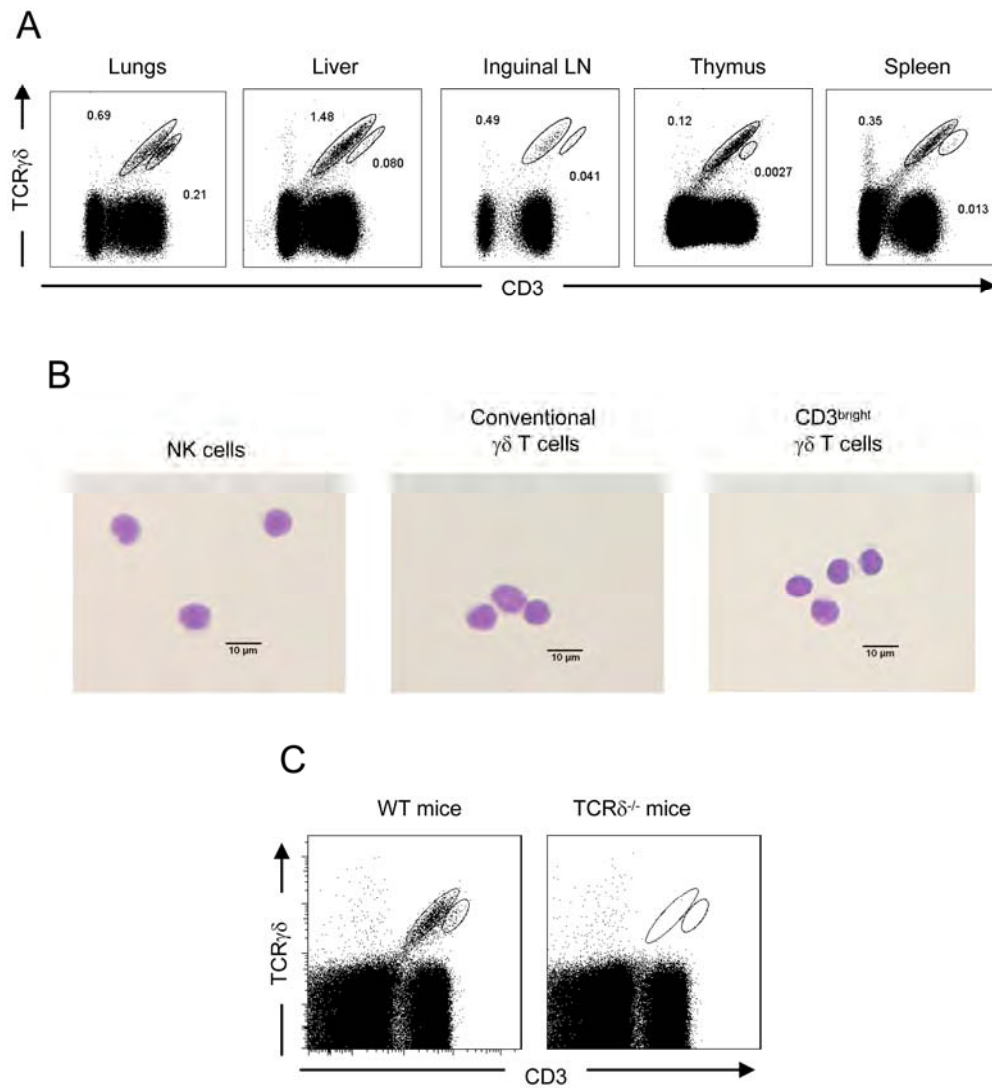


Figure S1: CD3^{bright} $\gamma\delta$ T cells are authentic $\gamma\delta$ T cells and conserved in different mouse backgrounds.

A, Cells from naïve Balb/c WT mice were analyzed by flow cytometry based on CD3 and TCR $\gamma\delta$ expression.

B, Morphology and Giemsa staining of cytopun FACS-sorted C57BL/6 WT lymphocyte populations.

C, Cells from naïve C57BL/6 WT and TCR $\delta^{-/-}$ mice were analyzed by flow cytometry based on CD3 and TCR $\gamma\delta$ expression.

Figure S2

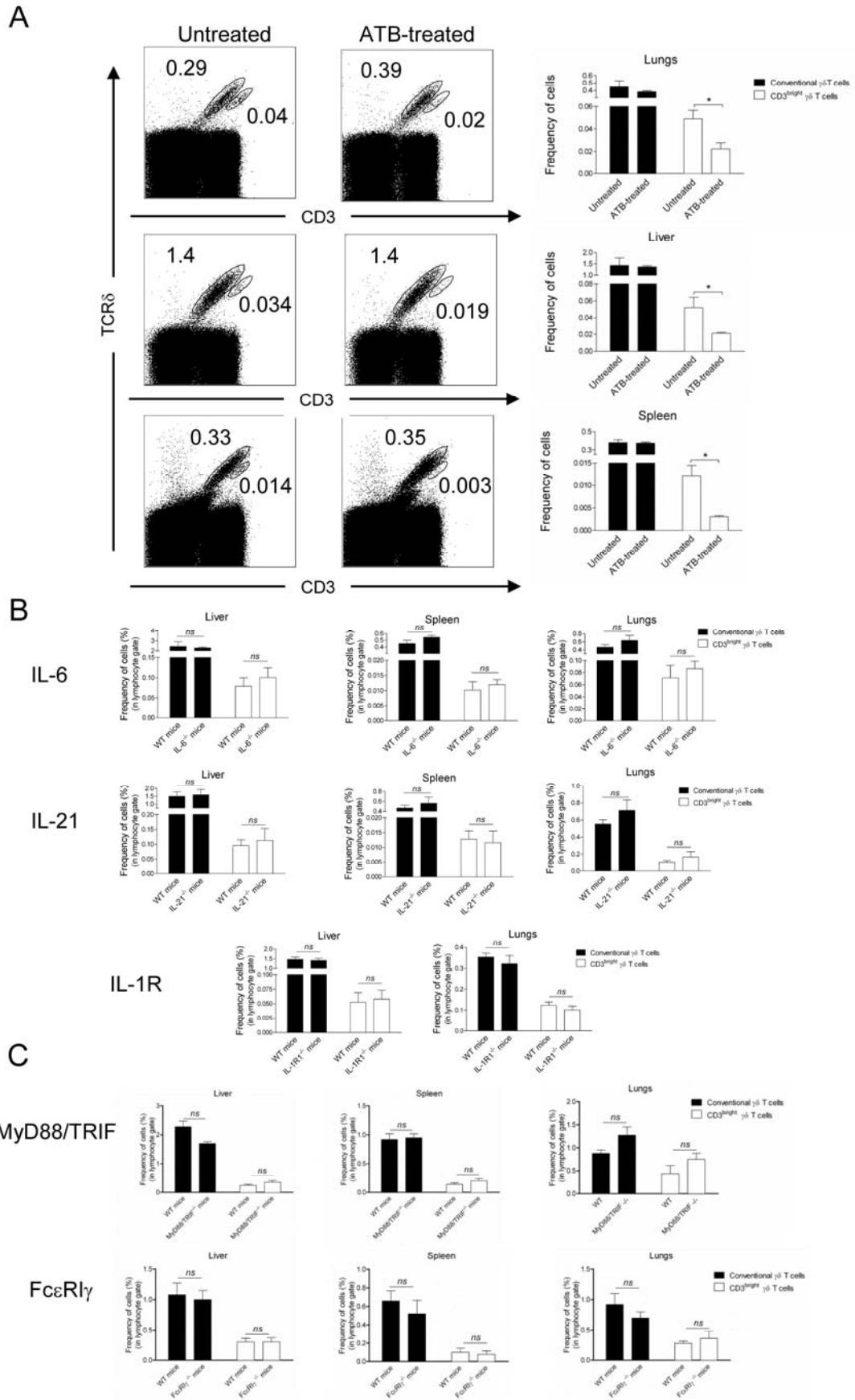


Figure S2: Factors influencing CD3^{bright} $\gamma\delta$ T cells homeostasis

A, Effect of chronic exposure to antibiotics on CD3^{bright} $\gamma\delta$ T cells. 5-week-old C57BL/6 mice were exposed three weeks with or without neomycin in the drinking water. Frequency of CD3^{bright} and conventional $\gamma\delta$ T cells in each group was evaluated by flow cytometry in different organs. Of note, antibiotic exposure did not impact on the total number of cells in each organ.

B, IL-6, IL21 and IL-1 do not impact on CD3^{bright} $\gamma\delta$ T cell frequency. Frequency of $\gamma\delta$ T subsets was analyzed by flow cytometry in WT vs IL-6^{-/-}, IL-21^{-/-} and IL-1R1^{-/-} mice. The percentage of $\gamma\delta$ T cell subsets is shown. The mean \pm SEM of $\gamma\delta$ T cell subset frequencies of one representative experiment out of two is shown ($n = 5-6$).

C, TLR-signalling and Fc ϵ RI γ are not involved in CD3^{bright} $\gamma\delta$ T cell homeostasis. Frequency of $\gamma\delta$ T subsets was analyzed by flow cytometry in WT vs MyD88/TRIF^{-/-} mice. The percentage of $\gamma\delta$ T cell subsets is shown. The mean \pm SEM of $\gamma\delta$ T cell subset frequencies of one representative experiment out of two is shown ($n = 5-6$).

Figure S3

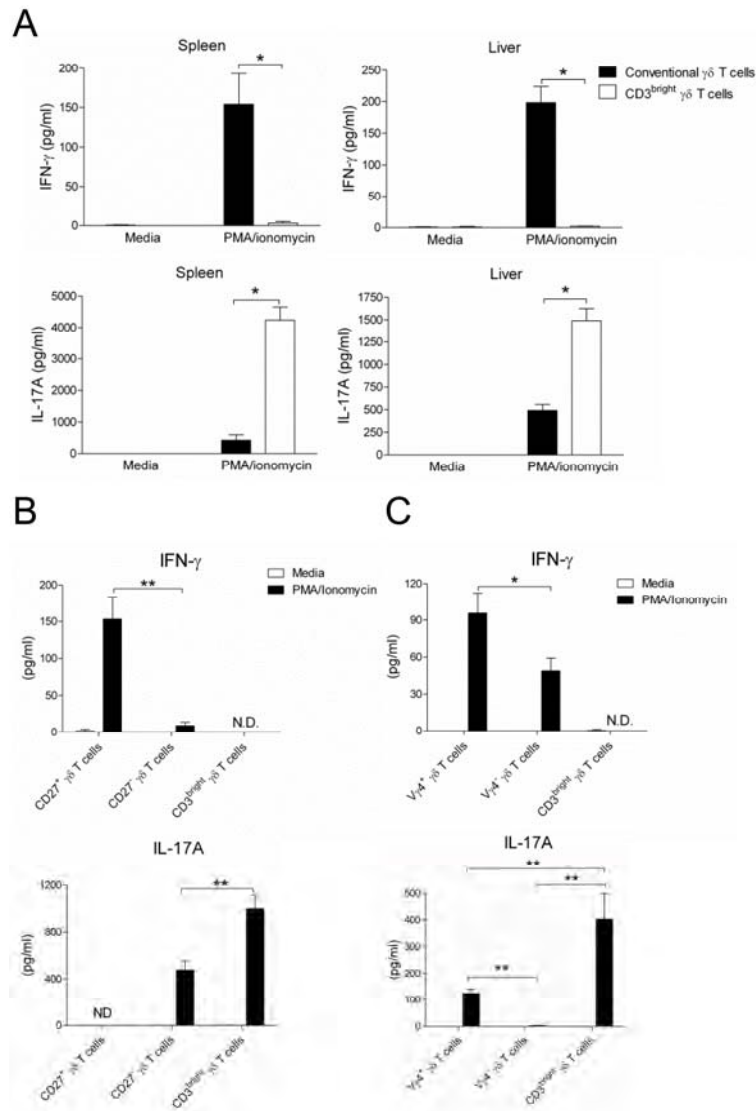


Figure S3: Capacity of CD3^{bright} $\gamma\delta$ T cells vs other $\gamma\delta$ T17 populations to produce IL-17A.

A, FACS-sorted splenic or hepatic $\gamma\delta$ T cell subsets were incubated with PMA (10 ng/ml) and ionomycin (1 μ g/ml). Data represent the mean \pm SEM of two independent experiments performed at least in duplicate. *, $p < 0.05$.

B and C, FACS-sorted pulmonary $\gamma\delta$ T cell subsets were incubated as in panel A. Cytokine production in the supernatants was measured by CBA. Data represent the mean \pm SEM of two independent experiments performed at least in duplicate. *, $p < 0.05$ and **, $p < 0.01$.

Figure S4

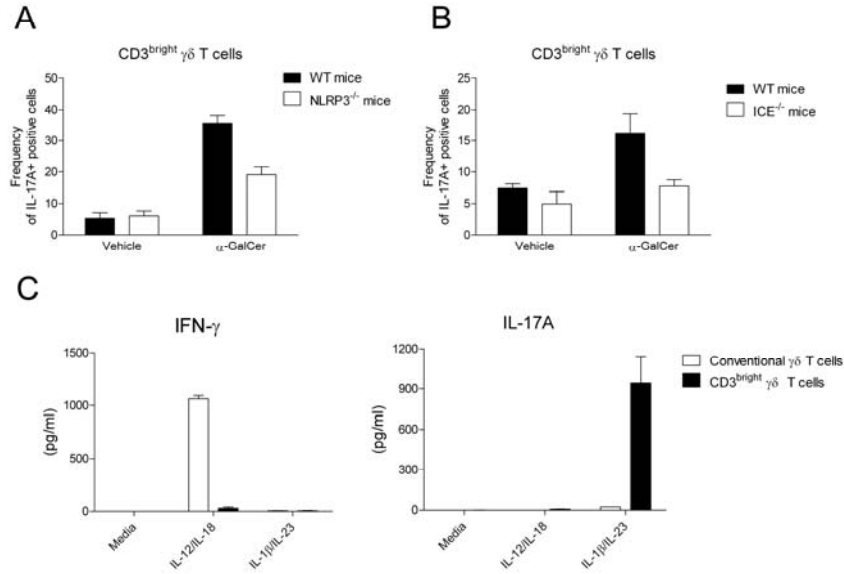


Figure S4: IL-17 production by CD3^{bright} γδ T cells requires NLRP3 inflammasome components *in vivo*.

A-B, WT, NLRP3^{-/-} (A) or ICE^{-/-} (B) mice were injected i.p. with vehicle or α-GalCer (2 μg/mouse) and were sacrificed after 2 hrs. Liver cells were treated with GolgiStop (2 μg/mouse) and were sacrificed after 2 hrs. Gated CD3^{bright} γδ T cells were screened for IL-17A production. The pooled mean ± SEM of two independent experiments for IL-17A-positive cells subsets is shown ($n = 5-6$).

C, FACS-sorted γδ T cell subsets were incubated or not with IL-12/IL-18 (50 pg/ml and 1 ng/ml respectively) or IL-1β/IL-23 (1 ng/ml for both) for 20 hrs. Cytokine production in the supernatants was measured by CBA. Data represent the mean ± SEM of two experiments performed in duplicate.

Figure S5

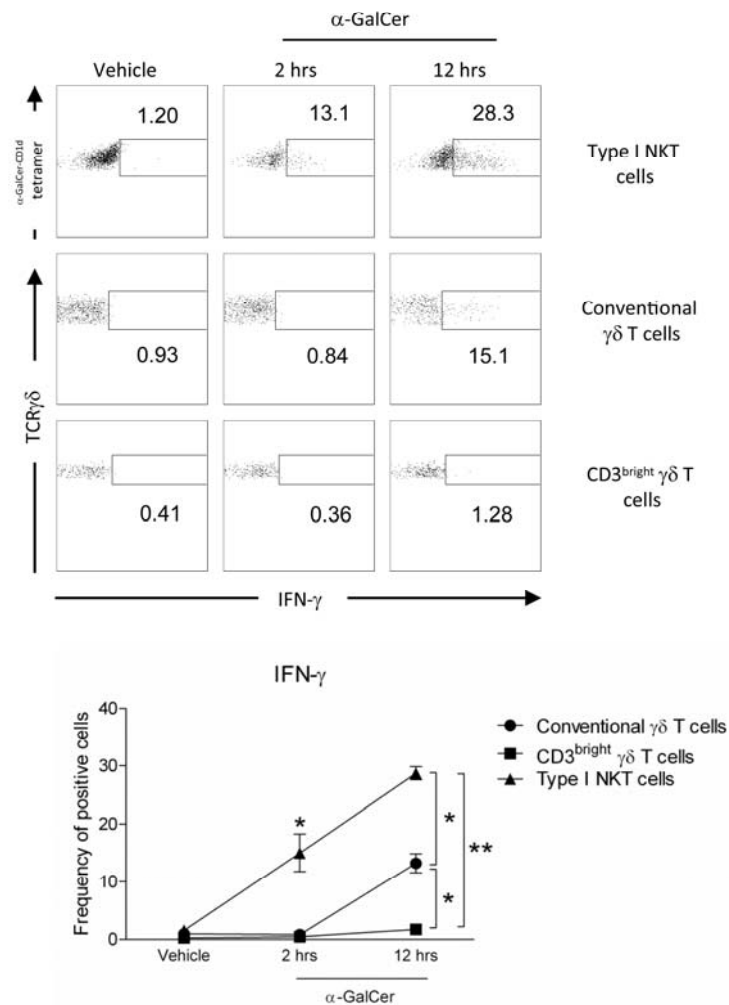


Figure S5: CD3^{bright} $\gamma\delta$ T cells do not produce IFN- γ following intranasal instillation of α -GalCer

WT mice were injected i.n. with vehicle or α -GalCer (500 ng/mouse) and were sacrificed at indicated times. Pulmonary cells were treated with GolgiPlug for another 2 hrs. Gated innate-like T cells were screened for IFN- γ production. One representative experiment out of three is shown (*upper panel*). The pooled mean \pm SEM of three independent experiments for IFN- γ -positive cells subsets is shown (6 mice/group/experiment) in the lower panel. *, p < 0.05 and **, p < 0.01.

Figure S6

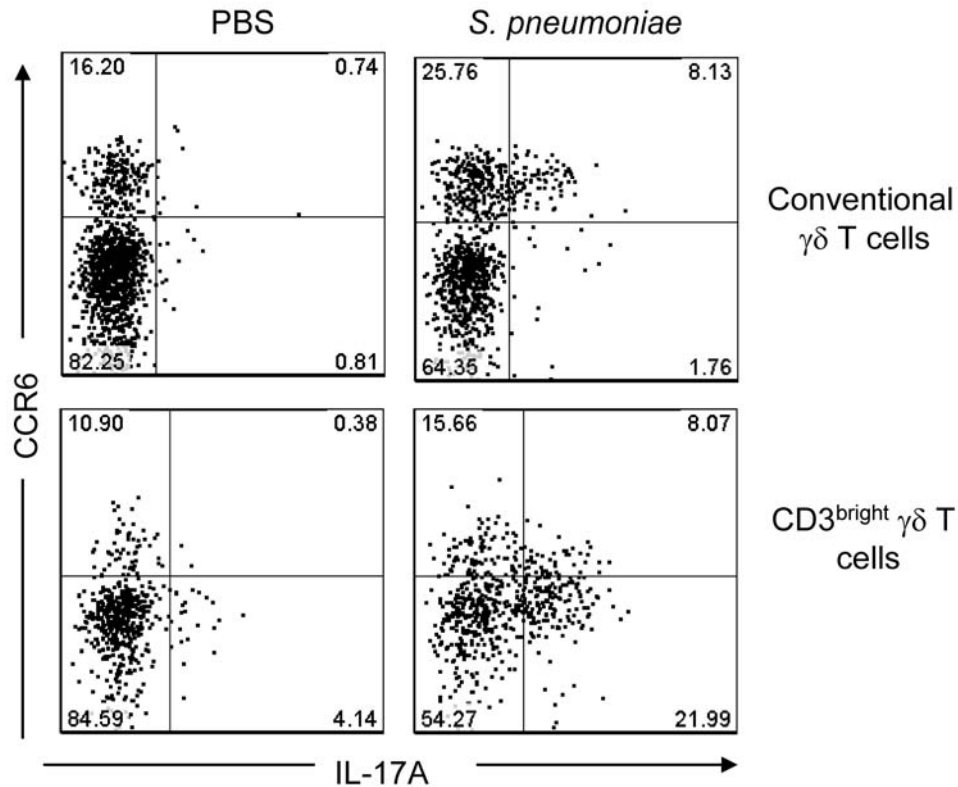


Figure S6: CCR6 expression on conventional, but not on CD3^{bright} $\gamma\delta$ T cells correlated with their ability to produce IL-17.

WT mice were infected i.n. with *S. pneumoniae* serotype 1 (2×10^6 bacteria) and were sacrificed at indicated time points. Gated pulmonary $\gamma\delta$ T cell subsets were screened for IL-17A production based on their CCR6 expression. One representative experiment out of two is shown. Gates were set based on the staining with isotype control.